

## **OLIGODEOXYNUCLEOTIDE AND ITS USE TO INDUCE AN IMMUNE RESPONSE**

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### **STATEMENT OF GOVERNMENT SUPPORT**

This invention was supported in part by Military Interdepartmental Purchase Request MM8926. The Government of the United States has certain rights in this invention.

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### **PRIORITY CLAIM**

This is a continuation-in-part of U.S. Application No. 09/958,713 filed on October 11, 2001, which claims the benefit of PCT Application No. PCT/US00/09839 filed on April 12, 2000, which claims priority to U.S. Provisional Patent Application No. 60/128,898 filed on April 12, 1999, all of which are incorporated herein by reference in their entirety.

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### **FIELD OF THE DISCLOSURE**

The present invention relates to the induction of an immune response, specifically to oligodeoxynucleotides including a CpG motif and their use in inducing an immune response.

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### **BACKGROUND**

Cells of the immune system recognize and are activated by conserved pathogen associated molecular patterns (PAMPs) in infectious agents. The unmethylated CpG dimers embedded in bacterial DNA, as well as certain synthetic oligodeoxynucleotides (ODNs) containing unmethylated CpG sequences (termed a CpG motif) that emulated them, are more frequent in the genomes of bacteria and viruses than vertebrates. Recent studies suggest that immune recognition of these motifs may contribute to the host's innate immune response (Klinman et al., Proc. Natl. Acad. Sci. USA 93: 2879, 1996;

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Yi et al, J. Immun. 157: 5394, 1996; Liang et al., J. Clin. Invest. 98:1119, 1996; Krieg et al., 374 Nature 374: 546, 1995).

In mice, CpG DNA induces proliferation in almost all (>95%) of B cells and increases immunoglobulin (Ig) secretion. This B-cell activation by CpG DNA is T-cell independent and antigen non-specific. In addition to its direct effects on B cells, CpG DNA has also been shown to activate cells of the immune system (see, for example, International Patent Applications WO 95/26204, WO 96/02555, WO 98/11211, WO 98/18810, WO 98/37919, WO 98/40100, WO 98/52581, PCT/US98/047703, and PCT/US99/07335; U.S. Patent No. 5,663,153).

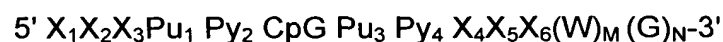
Although bacterial DNA and certain oligonucleotides can induce a murine immune response, little is known about the immunostimulatory capacity of these materials for the human immune system (Ballas et al., 157 *J. Immun.* 157: 1840 1996). In addition, differences in the responsiveness of human and murine B cells to certain stimuli render it difficult to extrapolate results obtained from mouse to man.

In view of the above, there exists a need for oligonucleotides that induce an immune response in humans. In addition, there is a need for methods utilizing CpG containing oligonucleotides in the treatment of human diseases.

### **BRIEF SUMMARY OF SPECIFIC EMBODIMENTS**

A substantially pure or isolated oligodeoxynucleotide (ODN) is disclosed herein that is at least about 16 nucleotides in length. The ODNs are referred to herein as D type ODNs, and are distinct from the previously described K type ODNs.

The oligodeoxynucleotide includes a sequence represented by the following formula:



wherein the central CpG motif is unmethylated, Pu is a purine nucleotide, Py is a pyrimidine nucleotide, X and W are any nucleotide, M is any integer from 0 to 10, and N is any integer from 4 to 10. In one embodiment,  $X_1X_2X_3$  and  $X_4X_5X_6$  are self-complementary. In another embodiment, at least two G's are included at the 5' end of the molecule, such that the oligodeoxynucleotide includes a sequence represented by the formula:



These oligodeoxynucleotides are also referred to as "D type" oligodeoxynucleotides. In one embodiment,  $\text{Pu}_1 \text{ Py}_2 \text{ CpG Pu}_3 \text{ Py}_4$  are phosphodiester bases. In one specific, non-limiting example,  $\text{Pu}_1$  is an adenine and  $\text{Py}_2$  is a tyrosine. In another specific, non-limiting example,  $\text{Pu}_3$  is an adenine and  $\text{Py}_4$  is a tyrosine.

These oligodeoxynucleotides stimulate cell types of the immune system to mount distinct immune responses. In one embodiment, the oligonucleotide induces production of a cytokine. Specific, non-limiting examples are interferon-gamma (IFN- $\gamma$ ), interferon-alpha (IFN- $\alpha$ ), IP-10, or IL-10. In another embodiment, a method is provided for activating a cell of the immune system. These cells include, but are not limited to, dendritic cells, natural killer (NK cells), and monocytes. Thus by employing D type ODN, the immune system can be manipulated to support specific therapeutic goals.

Also disclosed herein is a delivery complex for D type oligodeoxynucleotides and pharmacological composition comprising the D-type oligodeoxynucleotides.

### **BRIEF DESCRIPTION OF THE FIGURES**

**Fig. 1** is a set of bar graphs illustrating the response of PBMC to K and D ODN. PBMC from 35 donors were stimulated for 72 h with K or D ODN (3 mM). D19 (GGTGCATCGATGCAGGGGGG, SEQ ID NO: 1) and D29 (GGTGCACCGGTGCAGGGGGG, SEQ ID NO: 2) exemplify the response of PBMC to ODN that selectively induce IFN- $\gamma$ , whereas K3

(ATCGACTCTCGAGCGTTCTC, SEQ ID NO: 3) and K23 (TCGAGCGTTCT, SEQ ID NO: 4) exemplify ODN that induce IgM and IL-6 secretion and cell proliferation but little IFN- $\gamma$ . Control ODN have the CG dimer reversed or the C in the CG dimer replaced by a T; therefore, control D

- 5 (GGT**GCA**CGCGT**GCA**GGGGGG, SEQ ID NO: 5) and control K (TGCAGGCTTCTC, SEQ ID NO: 6). Bases in bold-face type are phosphorodiester, and those in normal type are phosphorothioate. Cytokine and immunoglobulin (Ig) concentrations in supernatants were determined by ELISA, and cell proliferation was assessed by [ $^3$ H] thymidine uptake. All
- 10 assays were done in triplicate. Statistical significance was determined by the nonparametric Mann-Whitney U test.

- Fig. 2** is several sets of sequences and bar graphs showing the parameters governing D ODN induced immune activation. Fig. 2A is a set of sequences and a bar graph demonstrating that D ODNs require an
- 15 unmethylated CpG. Fig. 2B is a set of sequences and a bar graph demonstrating that D ODNs require a phosphodiester backbone for optimal stimulation. Fig. 2C is a set of sequences and a bar graph demonstrating that a self complementary Pu<sub>1</sub>Py<sub>2</sub> CpG Pu<sub>3</sub>Py<sub>4</sub> increases the stimulation index. Fig. 2D is a set of sequences and a bar graph showing how size of an ODN
- 20 affects stimulation index. Fig. 2E is a set of sequences and a bar graph demonstrating that increased stimulation is obtained with a self complementary flanking region. Fig. 2F is a set of sequences and a bar graph demonstrating that 3' poly-G sequences can be used to improve activity of D ODNs. The ODN shown here are representative of 120 ODN used to
- 25 characterize the structural requirements of D ODN. CGs are underlined; immunostimulatory motifs are in bold; extended motifs are in italics; methylated bases have an asterisk; dots indicate identity; and shaded backgrounds identify phosphorodiester-linked bases. Important base changes in the sequence are circled. Data is expressed as stimulation
- 30 indices, representing the fold increase in cytokine secretion relative to unstimulated cells from the same donor. Bars represent the mean and SE of 20 different experiments. The ODN shown do not induce significant levels of

IgM or proliferation. Statistical significance was determined by the non-parametric Mann-Whitney U or nonparametric ANOVA: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

**Fig. 3** shows the cell type-dependent response to CpG ODN. Fold increase in IFN- $\gamma$  production by NK-92 cells and IL-6-secreting cell number by RPMI 8226 cells in response to ODN (3 mM for NK cells and 1 mM for B cells). The number of cells secreting IL-6 was determined by ELISPOT, and the secretion of IFN- $\gamma$  was determined at 72 h in culture supernatants by ELISA. Sequences: D19, D29 (see Fig. 1), D28 (GGTGCGTCGATGCAGGGGGG, SEQ ID NO: 7), K16 (TC-GACTCTCGAGCGTTCTC, SEQ ID NO: 8), K19 (ACTCTCGAGCGTTCTC, SEQ ID NO: 9), K101 (CTCGAGCGTTCT, SEQ ID NO: 10). Statistical significance determined by non-parametric Mann-Whitney U and non-parametric ANOVA tests.

**Fig. 4** shows the results of a study where PBMC from 8-20 normal human donors and 20 rhesus macaques were stimulated for 72 hours with a panel of "K", "D" or control ODN (3  $\mu$ M). IL-6 and IFN- $\alpha$  levels in culture supernatants were determined by ELISA while cell proliferation was assessed by [ $H$ ]<sup>3</sup> thymidine uptake. Note that D ODN induce the secretion of IFN $\alpha$  while K ODN induce cell proliferation and IL-6 production. All assays were performed in triplicate. Statistical significance was determined by ANOVA of log normalized data. \*  $p < 0.05$ ; \*\*  $p < 0.01$ .

**Fig. 5** shows the results of a study where PBMC from rhesus macaques (N = 12-20) were stimulated *in vitro* for 72 hours with a mixture of D19, D29 and D35 (1  $\mu$ M each) or K3 and K123 (1.5  $\mu$ M each). D122 and K163 were used in the control ODN mixture. Levels of IL-6 and IFN $\alpha$  in culture supernatants were measured by ELISA, while proliferation was measured by [ $H$ ]<sup>3</sup>-thymidine uptake. Statistical significance was determined by ANOVA of the normalized data. \*\*  $p < 0.01$ .

**Fig. 6** shows the results of a study where Macaques (3/group) were immunized on day 0 s.c. with 4  $\mu$ g of OVA plus 125  $\mu$ g of alum and 250  $\mu$ g of a mixture of (D19 + D29), (K3 + K23) or control (AA3M) ODN. Monkeys were boosted 12 weeks later (black arrow). Serum IgG anti-OVA titers were

determined by ELISA. Values represent the geometric mean titer  $\pm$  SEM. The anti-OVA IgG titers in the group that received "D" ODN are significantly higher ( $p < 0.01$ ).

**Fig. 7** shows the results of a study where Rhesus macaques were primed subcutaneously (s.c.) with 250  $\mu$ g of alum-adjuvanted HKLV alone (N=6) or combined with 500  $\mu$ g of a mixture of (D19, D29 and D35) (N=5) or (K3 and K123) (N=5) and boosted 4 weeks later. On week 14, the monkeys were challenged on the opposite forehead with  $10^7$  metacyclic promastigotes. The average size of the resultant lesions is shown as the mean area (calculated as  $\text{mean diameter}/2)^2 \times \pi$ ). Macaques immunized with HKLV-ALUM-"D"ODN had significantly smaller lesions ( $p < 0.01$ ).

### DETAILED DESCRIPTION OF SEVERAL EMBODIMENTS

#### I. Abbreviations

- A:** adenine
- Ab:** antibody
- APC:** antigen presenting cell
- C:** cytosine
- APC:** antigen presenting cell
- CpG ODN:** an oligodeoxynucleotide (either a D or a K type) including a CpG motif.
- DC:** dendritic cell
- FCS:** fetal calf serum
- G:** guanine
- h:** hour
- HKLV:** heat-killed leishmania vaccine
- IFN- $\alpha$ :** interferon alpha
- IFN- $\gamma$ :** interferon gamma
- IL-10:** interleukin 10
- mm:** millimeter

**mRNA:** messenger ribonucleic acid.

**ODN:** oligodeoxynucleotide

**Pu:** purine

**Py:** pyrimidine

5 **s.c.:** subcutaneous

**T:** thymine

**µg:** microgram

## II. Terms

10 Unless otherwise noted, technical terms are used according to  
conventional usage. Definitions of common terms in molecular biology may  
be found in Benjamin Lewin, *Genes V*, published by Oxford University Press,  
1994 (ISBN 0-19-854287-9); Kendrew *et al.* (eds.), *The Encyclopedia of*  
15 *Molecular Biology*, published by Blackwell Science Ltd., 1994 (ISBN 0-632-  
02182-9); and Robert A. Meyers (ed.), *Molecular Biology and Biotechnology: a*  
*Comprehensive Desk Reference*, published by VCH Publishers, Inc., 1995  
(ISBN 1-56081-569-8).

Unless otherwise explained, all technical and scientific terms used  
herein have the same meaning as commonly understood by one of ordinary  
20 skill in the art to which this invention belongs. The singular terms "a," "an,"  
and "the" include plural referents unless context clearly indicates otherwise.  
Similarly, the word "or" is intended to include "and" unless the context clearly  
indicates otherwise. The term "comprises" means "includes." It is further to be  
understood that all base sizes or amino acid sizes, and all molecular weight or  
25 molecular mass values, given for nucleic acids or polypeptides are  
approximate, and are provided for description. Although methods and  
materials similar or equivalent to those described herein can be used in the  
practice or testing of the present invention, suitable methods and materials are  
described below. In case of conflict, the present specification, including  
30 explanations of terms, will control. In addition, the materials, methods, and  
examples are illustrative only and not intended to be limiting.

In order to facilitate review of the various embodiments of the invention, the following explanations of specific terms are provided:

**Allergen:** A substance that can induce an allergic or asthmatic response in a susceptible subject. The list of allergens is enormous and can include pollens, insect venoms, animal dander dust, fungal spores and drugs (e.g. penicillin). Examples of natural, animal and plant allergens include proteins specific to the following genera: Canine (*Canis familiaris*); Dermatophagoides (e.g. *Dermatophagoides farinae*); Felis (*Felis domesticus*); Ambrosia (*Ambrosia artemisiifolia*); Lolium (e.g. *Lolium perenne* or *Lolium multiflorum*); Cryptomeria (*Cryptomeria japonica*); Alternaria (*Alternaria alternata*); Alder; Alnus (*Alnus gultinosa*); Betula (*Betula verrucosa*); Quercus (*Quercus alba*); Olea (*Olea europa*); Artemisia (*Artemisia vulgaris*); Plantago (e.g. *Plantago lanceolata*); Parietaria (e.g. *Parietaria officinalis* or *Parietaria judaica*); Blattella (e.g. *Blattella germanica*); Apis (e.g. *Apis multiflorum*); Cupressus (e.g. *Cupressus sempervirens*, *Cupressus arizonica* and *Cupressus macrocarpa*); Juniperus (e.g. *Juniperus sabinoideis*, *Juniperus virginiana*, *Juniperus communis* and *Juniperus ashei*); Thuya (e.g. *Thuya orientalis*); Chamaecyparis (e.g. *Chamaecyparis obtusa*); Periplaneta (e.g. *Periplaneta americana*); Agropyron (e.g. *Agropyron repens*); Secale (e.g. *Secale cereale*); Triticum (e.g. *Triticum aestivum*); Dactylis (e.g. *Dactylis glomerata*); Festuca (e.g. *Festuca elatior*); Poa (e.g. *Poa pratensis* or *Poa compressa*); Avena (e.g. *Avena sativa*); Holcus (e.g. *Holcus lanatus*); Anthoxanthum (e.g. *Anthoxanthum odoratum*); Arrhenatherum (e.g. *Arrhenatherum elatius*); Agrostis (e.g. *Agrostis alba*); Phleum (e.g. *Phleum pratense*); Phalaris (e.g. *Phalaris arundinacea*); Paspalum (e.g. *Paspalum notatum*); Sorghum (e.g. *Sorghum halepensis*); and Bromus (e.g. *Bromus inermis*). The term "allergy" refers to acquired hypersensitivity to a substance (allergen). An "allergic reaction" is the response of an immune system to an allergen in a subject allergic to the allergen. Allergic conditions include eczema, allergic rhinitis or coryza, hay fever, bronchial asthma, urticaria (hives) and food allergies, and other atopic conditions.



**Animal:** Living multi-cellular vertebrate organisms, a category that includes, for example, mammals and birds. The term mammal includes both human and non-human mammals. Similarly, the term "subject" includes both human and veterinary subjects.

5       **Antigen:** A compound, composition, or substance that can stimulate the production of antibodies or a T-cell response in an animal, including compositions that are injected or absorbed into an animal. An antigen reacts with the products of specific humoral or cellular immunity, including those induced by heterologous immunogens. The term "antigen" includes all related  
10       antigenic epitopes.

**Anti-infectious agent:** A substance (such as a chemical compound, protein, antisense oligonucleotide, or other molecule) of use in treating infection of a subject. Anti-infectious agents include, but are not limited to, anti-fungals, anti-virals, and antibiotics.

15       **Antisense, Sense, and Antigene:** Double-stranded DNA (dsDNA) has two strands, a 5' -> 3' strand, referred to as the plus strand, and a 3' -> 5' strand (the reverse compliment), referred to as the minus strand. Because RNA polymerase adds nucleic acids in a 5' -> 3' direction, the minus strand of the DNA serves as the template for the RNA during transcription. Thus, the  
20       RNA formed will have a sequence complementary to the minus strand and identical to the plus strand (except that U is substituted for T).

      Antisense molecules are molecules that are specifically hybridizable or specifically complementary to either RNA or the plus strand of DNA. Sense molecules are molecules that are specifically hybridizable or specifically  
25       complementary to the minus strand of DNA. Antigene molecules are either antisense or sense molecules directed to a dsDNA target. In one embodiment, an antisense molecule specifically hybridizes to a target mRNA and inhibits transcription of the target mRNA.

**Asthma:** A disorder of the respiratory system characterized by  
30       inflammation, narrowing of the airways and increased reactivity of the airways to inhaled agents. Asthma is frequently, although not exclusively associated with atopic or allergic symptoms.

**Autoimmune disorder:** A disorder in which the immune system produces an immune response (e.g. a B cell or a T cell response) against an endogenous antigen, with consequent injury to tissues.

**CpG or CpG motif:** A nucleic acid having a cytosine followed by a  
5 guanine linked by a phosphate bond in which the pyrimidine ring of the  
cytosine is unmethylated. The term "methylated CpG" refers to the  
methylation of the cytosine on the pyrimidine ring, usually occurring the 5-  
position of the pyrimidine ring. A CpG motif is a pattern of bases that include  
an unmethylated central CpG surrounded by at least one base flanking (on  
10 the 3' and the 5' side of) the central CpG. Without being bound by theory, the  
bases flanking the CpG confer part of the activity to the CpG  
oligodeoxynucleotide. A CpG oligonucleotide is an oligonucleotide that is at  
least about ten nucleotides in length and includes an unmethylated CpG.  
CpG oligonucleotides include both D and K type oligodeoxynucleotides (see  
15 below). CpG oligodeoxynucleotides are single-stranded. The entire CpG  
oligodeoxynucleotide can be unmethylated or portions may be unmethylated.  
In one embodiment, at least the C of the 5' CG 3' is unmethylated.

**Cancer:** A malignant neoplasm that has undergone characteristic  
anaplasia with loss of differentiation, increase rate of growth, invasion of  
20 surrounding tissue, and is capable of metastasis. For example, thyroid cancer  
is a malignant neoplasm that arises in or from thyroid tissue, and breast  
cancer is a malignant neoplasm that arises in or from breast tissue (such as a  
ductal carcinoma). Residual cancer is cancer that remains in a subject after  
any form of treatment given to the subject to reduce or eradicate thyroid  
25 cancer. Metastatic cancer is a cancer at one or more sites in the body other  
than the site of origin of the original (primary) cancer from which the  
metastatic cancer is derived.

**Chemotherapy; chemotherapeutic agents:** As used herein, any  
chemical agent with therapeutic usefulness in the treatment of diseases  
30 characterized by abnormal cell growth. Such diseases include tumors,  
neoplasms, and cancer as well as diseases characterized by hyperplastic  
growth such as psoriasis. In one embodiment, a chemotherapeutic agent is

an agent of use in treating neoplasms such as solid tumors. In one embodiment, a chemotherapeutic agent is radioactive molecule. One of skill in the art can readily identify a chemotherapeutic agent of use (e.g. see Slapak and Kufe, *Principles of Cancer Therapy*, Chapter 86 in Harrison's Principles of Internal Medicine, 14th edition; Perry et al., *Chemotherapy*, Ch. 17 in Abeloff, *Clinical Oncology* 2<sup>nd</sup> ed., © 2000 Churchill Livingstone, Inc; Baltzer L, Berkery R (eds): *Oncology Pocket Guide to Chemotherapy*, 2nd ed. St. Louis, Mosby-Year Book, 1995; Fischer DS, Knobf MF, Durivage HJ (eds): *The Cancer Chemotherapy Handbook*, 4th ed. St. Louis, Mosby-Year Book, 1993).

**Cytokine:** Proteins made by cells that affect the behavior of other cells, such as lymphocytes. In one embodiment, a cytokine is a chemokine, a molecule that affects cellular trafficking.

**Dendritic cell (DC):** Dendritic cells are the principle antigen presenting cells (APCs) involved in primary immune responses. Their major function is to obtain antigen in tissues, migrate to lymphoid organs and present the antigen in order to activate T cells.

When an appropriate maturational cue is received, DC are signaled to undergo rapid morphological and physiological changes that facilitate the initiation and development of immune responses. Among these are the up-regulation of molecules involved in antigen presentation; production of pro-inflammatory cytokines, including IL-12, key to the generation of Th1 responses; and secretion of chemokines that help to drive differentiation, expansion, and migration of surrounding naive Th cells. Collectively, these up-regulated molecules facilitate the ability of DC to coordinate the activation and effector function of other surrounding lymphocytes that ultimately provide protection for the host. Although the process of DC maturation is commonly associated with events that lead to the generation of adaptive immunity, many stimuli derived from the innate branch of the immune system are also capable of activating DC to initiate this process. In this manner, DC provide a link between the two branches of the immune response, in which their initial activation during the innate response can influence both the nature and

magnitude of the ensuing adaptive response. A dendritic cell precursor is a cell that matures into an antigen presenting dendritic cell. In one embodiment, a dendritic cell is a plasmacytoid dendritic cell.

**Differentiation:** The process by which cells become more specialized to perform biological functions, and differentiation is a property that is totally or partially lost by cells that have undergone malignant transformation. For example, dendritic cell precursors undergo maturation to become APCs.

**Epitope:** An antigenic determinant. These are particular chemical groups or peptide sequences on a molecule that are antigenic, i.e. that elicit a specific immune response. An antibody binds a particular antigenic epitope.

**Functionally Equivalent:** Sequence alterations, for example in a D type ODN, that yield the same results as described herein. Such sequence alterations can include, but are not limited to, deletions, base modifications, mutations, labeling, and insertions.

**Immune response:** A response of a cell of the immune system, such as a B cell, T cell to a stimulus. In one embodiment, the response is specific for a particular antigen (an "antigen-specific response").

**Immune system deficiency:** A disease or disorder in which the subject's immune system is not functioning in normal capacity or in which it would be useful to boost a subject's immune response. Immune system deficiencies include those diseases or disorders in which the immune system is not functioning at normal capacity, or in which it would be useful to boost the immune system response. In one specific, non-limiting example, a subject with an immune system deficiency has a tumor or cancer (e.g. tumors of the brain, lung (e.g. small cell and non-small cell), ovary, breast, prostate, colon, as well as other carcinomas and sarcomas).

**Infectious agent:** An agent that can infect a subject, including, but not limited to, viruses, bacteria, and fungi.

Examples of infectious virus include: *Retroviridae*; *Picornaviridae* (for example, polio viruses, hepatitis A virus; enteroviruses, human coxsackie viruses, rhinoviruses, echoviruses); *Calciviridae* (such as strains that cause gastroenteritis); *Togaviridae* (for example, equine encephalitis viruses, rubella

- viruses); *Flaviridae* (for example, dengue viruses, encephalitis viruses, yellow fever viruses); *Coronaviridae* (for example, coronaviruses); *Rhabdoviridae* (for example, vesicular stomatitis viruses, rabies viruses); *Filoviridae* (for example, ebola viruses); *Paramyxoviridae* (for example, parainfluenza viruses, mumps virus, measles virus, respiratory syncytial virus); *Orthomyxoviridae* (for example, influenza viruses); *Bunyaviridae* (for example, Hantaan viruses, bunga viruses, phleboviruses and Nairo viruses); *Arena viridae* (hemorrhagic fever viruses); *Reoviridae* (e.g., reoviruses, orbiviruses and rotaviruses); *Bimaviridae*; *Hepadnaviridae* (Hepatitis B virus); *Parvoviridae* (parvoviruses);
- 10 *Papovaviridae* (papilloma viruses, polyoma viruses); *Adenoviridae* (most adenoviruses); *Herpesviridae* (herpes simplex virus (HSV) 1 and HSV-2, varicella zoster virus, cytomegalovirus (CMV), herpes viruses); *Poxviridae* (variola viruses, vaccinia viruses, pox viruses); and *Iridoviridae* (such as African swine fever virus); and unclassified viruses (for example, the
- 15 etiological agents of Spongiform encephalopathies, the agent of delta hepatitis (thought to be a defective satellite of hepatitis B virus), the agents of non-A, non-B hepatitis (class 1=internally transmitted; class 2=parenterally transmitted (i.e., Hepatitis C); Norwalk and related viruses, and astroviruses).

- Examples of infectious bacteria include: *Helicobacter pylori*, *Borelia burgdorferi*, *Legionella pneumophila*, *Mycobacteria* sps (such as *M. tuberculosis*, *M. avium*, *M. intracellulare*, *M. kansaii*, *M. gordonae*), *Staphylococcus aureus*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Listeria monocytogenes*, *Streptococcus pyogenes* (Group A *Streptococcus*), *Streptococcus agalactiae* (Group B *Streptococcus*), *Streptococcus* (*viridans* group), *Streptococcus faecalis*, *Streptococcus bovis*, *Streptococcus* (*anaerobic* sps.), *Streptococcus pneumoniae*, *pathogenic Campylobacter* sp., *Enterococcus* sp., *Haemophilus influenzae*, *Bacillus anthracis*, *Corynebacterium diphtheriae*, *Corynebacterium* sp., *Erysipelothrix rhusiopathiae*, *Clostridium perfringens*, *Clostridium tetani*, *Enterobacter*
- 25 *aerogenes*, *Klebsiella pneumoniae*, *Pasteurella multocida*, *Bacteroides* sp., *Fusobacterium nucleatum*, *Streptobacillus moniliformis*, *Treponema pallidum*, *Treponema pertenue*, *Leptospira*, and *Actinomyces israeli*.
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Examples of infectious fungi include, but are not limited to, *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Coccidioides immitis*, *Blastomyces dermatitidis*, *Chlamydia trachomatis*, *Candida albicans*.

Other infectious organisms (such as protists) include: *Plasmodium falciparum* and *Toxoplasma gondii*.

**Interferon alpha:** At least 23 different variants of IFN- $\alpha$  are known. The individual proteins have molecular masses between 19-26 kDa and consist of proteins with lengths of 156-166 and 172 amino acids. All IFN- $\alpha$  subtypes possess a common conserved sequence region between amino acid positions 115-151 while the amino-terminal ends are variable. Many IFN- $\alpha$  subtypes differ in their sequences at only one or two positions. Naturally occurring variants also include proteins truncated by 10 amino acids at the carboxy-terminal end.

There are at least 23 different IFN- $\alpha$  genes. They have a length of 1-2 kb and are clustered on human chromosome 9p22. Based upon the structures two types of IFN-alpha genes, designated class I and II, are distinguished. They encode proteins of 156-166 and 172 amino acids, respectively.

IFN- $\alpha$  is assayed by a cytopathic effect reduction test employing human and bovine cell lines. Minute amounts of IFN- $\alpha$  can be assayed also by detection of the Mx protein specifically induced by this interferon. A sandwich ELISA employing bi-specific monoclonal antibodies for rapid detection is also available.

**Interferon gamma:** IFN- $\gamma$  is a dimeric protein with subunits of 146 amino acids. The protein is glycosylated at two sites, and the pI is 8.3-8.5. IFN- $\gamma$  is synthesized as a precursor protein of 166 amino acids including a secretory signal sequence of 23 amino acids. Two molecular forms of the biologically active protein of 20 and 25 kDa have been described. Both of them are glycosylated at position 25. The 25 kDa form is also glycosylated at position 97. The observed differences of natural IFN- $\gamma$  with respect to molecular mass and charge are due to variable glycosylation patterns. 40-60 kDa forms observed under non-denaturing conditions are dimers and

tetramers of IFN- $\gamma$ . The human gene has a length of approximately 6 kb. It contains four exons and maps to chromosome 12q24.1.

IFN- $\gamma$  can be detected by sensitive immunoassays, such as an ELSA test that allows detection of individual cells producing IFN- $\gamma$ . Minute amounts of IFN- $\gamma$  can be detected indirectly by measuring IFN-induced proteins such as Mx protein. The induction of the synthesis of IP-10 has been used also to measure IFN-gamma concentrations. In addition, bioassays can be used to detect IFN- $\gamma$ , such as an assay that employs induction of indoleamine 2,3-dioxygenase activity in 2D9 cells.

**Interferon Inducible Protein 10:** A cytokine that is 98 amino acids in length that has homology to platelet factor-4, and is a chemokine. The human IP-10 genes contains four exons and maps to chromosome 4q12-21.

**Interleukin-10:** IL-10 is a homodimeric protein with subunits having a length of 160 amino acids that is a cytokine. Human IL-10 shows 73 percent amino acid homology with murine IL-10. The human IL-10 gene contains four exons.

IL10 inhibits the synthesis of a number of cytokines such as IL-2 and IFN- $\gamma$  in Th1 subpopulations of T-cells but not of Th2. IL10 can be detected with an ELISA assay. In addition, the murine mast cell line D36 can be used to bioassay human IL10. The intracellular factor can be detected also by flow cytometry.

**Isolated:** An "isolated" biological component (such as a nucleic acid, peptide or protein) has been substantially separated, produced apart from, or purified away from other biological components in the cell of the organism in which the component naturally occurs, i.e., other chromosomal and extrachromosomal DNA and RNA, and proteins. Nucleic acids, peptides and proteins which have been "isolated" thus include nucleic acids and proteins purified by standard purification methods. The term also embraces nucleic acids, peptides and proteins prepared by recombinant expression in a host cell as well as chemically synthesized nucleic acids.

**Leukocyte:** Cells in the blood, also termed "white cells," that are involved in defending the body against infective organisms and foreign

substances. Leukocytes are produced in the bone marrow. There are 5 main types of white blood cell, subdivided between 2 main groups:

polymorphonuclear leukocytes (neutrophils, eosinophils, basophils) and mononuclear leukocytes (monocytes and lymphocytes). When an infection is present, the production of leukocytes increases.

**Mammal:** This term includes both human and non-human mammals. Similarly, the term "subject" includes both human and veterinary subjects.

**Maturation:** The process in which an immature cell, such as dendritic cell, changes in form or function to become a functional mature cell, such as an APC.

**Neoplasm:** An abnormal cellular proliferation, which includes benign and malignant tumors, as well as other proliferative disorders.

**Nucleic acid:** A deoxyribonucleotide or ribonucleotide polymer in either single or double stranded form, and unless otherwise limited, encompasses known analogues of natural nucleotides that hybridize to nucleic acids in a manner similar to naturally occurring nucleotides.

**Oligonucleotide** or "oligo": Multiple nucleotides (i.e. molecules comprising a sugar (e.g. ribose or deoxyribose) linked to a phosphate group and to an exchangeable organic base, which is either a substituted pyrimidine (Py) (e.g. cytosine (C), thymine (T) or uracil (U)) or a substituted purine (Pu) (e.g. adenine (A) or guanine (G)). The term "oligonucleotide" as used herein refers to both oligoribonucleotides (ORNs) and oligodeoxyribonucleotides (ODNs). The term "oligonucleotide" also includes oligonucleosides (i.e. an oligonucleotide minus the phosphate) and any other organic base polymer. Oligonucleotides can be obtained from existing nucleic acid sources (e.g. genomic or cDNA), but are preferably synthetic (e.g. produced by oligonucleotide synthesis).

A "stabilized oligonucleotide" is an oligonucleotide that is relatively resistant to *in vivo* degradation (for example via an exo- or endo-nuclease). In one embodiment, a stabilized oligonucleotide has a modified phosphate backbone. One specific, non-limiting example of a stabilized oligonucleotide has a phosphorothioate modified phosphate backbone (wherein at least one of



the phosphate oxygens is replaced by sulfur). Other stabilized oligonucleotides include: nonionic DNA analogs, such as alkyl- and aryl-phosphonates (in which the charged phosphonate oxygen is replaced by an alkyl or aryl group), phosphodiester and alkylphosphotriesters, in which the charged oxygen moiety is alkylated. Oligonucleotides which contain a diol, such as tetraethyleneglycol or hexaethyleneglycol, at either or both termini have also been shown to be substantially resistant to nuclease degradation.

An "immunostimulatory oligonucleotide," "immunostimulatory CpG containing oligodeoxynucleotide," "CpG ODN," refers to an oligodeoxynucleotide, which contains a cytosine, guanine dinucleotide sequence and stimulates (e.g. has a mitogenic effect or induces cytokine production) vertebrate immune cells. The cytosine, guanine is unmethylated.

An "oligonucleotide delivery complex" is an oligonucleotide associated with (e.g. ionically or covalently bound to; or encapsulated within) a targeting means (e.g. a molecule that results in a higher affinity binding to a target cell (e.g. B-cell or natural killer (NK) cell) surface and/or increased cellular uptake by target cells). Examples of oligonucleotide delivery complexes include oligonucleotides associated with: a sterol (e.g. cholesterol), a lipid (e.g. cationic lipid, virosome or liposome), or a target cell specific binding agent (e.g. a ligand recognized by a target cell specific receptor). Preferred complexes must be sufficiently stable in vivo to prevent significant uncoupling prior to internalization by the target cell. However, the complex should be cleavable or otherwise accessible under appropriate conditions within the cell so that the oligonucleotide is functional. (Gursel, J. Immunol. 167: 3324, 2001)

**Pharmaceutical agent or drug:** A chemical compound or composition capable of inducing a desired therapeutic or prophylactic effect when properly administered to a subject. Pharmaceutical agents include, but are not limited to, chemotherapeutic agents and anti-infective agents.

**Pharmaceutically acceptable carriers:** The pharmaceutically acceptable carriers useful in this invention are conventional. *Remington's Pharmaceutical Sciences*, by E. W. Martin, Mack Publishing Co., Easton, PA,

15th Edition (1975), describes compositions and formulations suitable for pharmaceutical delivery of the fusion proteins herein disclosed.

In general, the nature of the carrier will depend on the particular mode of administration being employed. For instance, parenteral formulations usually comprise injectable fluids that include pharmaceutically and physiologically acceptable fluids such as water, physiological saline, balanced salt solutions, aqueous dextrose, glycerol or the like as a vehicle. For solid compositions (e.g., powder, pill, tablet, or capsule forms), conventional non-toxic solid carriers can include, for example, pharmaceutical grades of mannitol, lactose, starch, or magnesium stearate. In addition to biologically-neutral carriers, pharmaceutical compositions to be administered can contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate.

**Preventing or treating a disease:** "Preventing" a disease refers to inhibiting the full development of a disease, for example in a person who is known to have a predisposition to a disease such as an autoimmune disorder. An example of a person with a known predisposition is someone with a history of diabetes in the family, or who has been exposed to factors that predispose the subject to a condition, such as lupus or rheumatoid arthritis. "Treatment" refers to a therapeutic intervention that ameliorates a sign or symptom of a disease or pathological condition after it has begun to develop.

**Purified:** The term purified does not require absolute purity; rather, it is intended as a relative term. Thus, for example, a purified peptide preparation is one in which the peptide or protein is more enriched than the peptide or protein is in its natural environment within a cell. Preferably, a preparation is purified such that the protein or peptide represents at least 50% of the total peptide or protein content of the preparation.

**Self-complementary nucleic acid sequence:** A nucleic acid sequence that can form Watson-Crick base pairs. The four bases characteristic of deoxyribonucleic unit of DNA are the purines (adenine and guanine) and the pyrimidines (cytosine and thymine). Adenine pairs with

thymine via two hydrogen bonds, while guanine pairs with cytosine via three hydrogen bonds. If a nucleic acid sequence includes two or more bases in sequence that can form hydrogen bonds with two or more other bases in the same nucleic acid sequence, then the nucleic acid includes a self-

- 5 complementary sequence. In several embodiments, a self-complementary nucleic acid sequence includes 3, 4, 5, 6 or more bases that could form hydrogen bonds with 3, 4, 5, 6 or more bases, respectively, of the same nucleic acid sequence.

- Therapeutically effective dose:** A dose sufficient to prevent  
10 advancement, or to cause regression of the disease, or which is capable of relieving symptoms caused by the disease, such as pain or swelling.

- Vaccine:** A preparation of attenuated microorganisms (including but not limited to bacteria and viruses), living microorganisms, antigen, or killed microorganisms, administered for the prevention, amelioration or treatment of  
15 infectious disease.

A. CpG Oligodeoxynucleotides

- K type CpG ODNs have been previously described. K ODNs which exhibit the greatest immunostimulatory activity share specific characteristics.  
20 These characteristics differ from those of the Formula II or D ODN (see below). In addition, K ODN have specific effects on the cells of the immune system, which differ from the effects of D ODN. For example, K ODN stimulate proliferation of B cells and stimulate the production of IL-6.

- The K ODNs at least about 10 nucleotides and include a sequence  
25 represented by either Formula I:



wherein the central CpG motif is unmethylated, W is A or T, and N<sub>1</sub>, N<sub>2</sub>, N<sub>3</sub>, N<sub>4</sub>, N<sub>5</sub>, and N<sub>6</sub> are any nucleotides.

- These Formula I or K ODN, stimulate B cell proliferation and the  
30 secretion of IgM and IL-6, processes involved in the body's humoral immunity,

such as the production of antibodies against foreign antigens. In one embodiment, the K ODNs induce a humoral immune response.

In one embodiment, K type oligonucleotides of the formula



5 contain a phosphate backbone modification. In one specific, non-limiting example, the phosphate backbone modification is a phosphorothioate backbone modification (i.e., one of the non-bridging oxygens is replaced with sulfur, as set forth in International Patent Application WO 95/26204, herein incorporated by reference). In one embodiment, K ODNs have a  
10 phosphorothioate backbone, and at least one unmethylated CpG dinucleotide. Eliminating the CpG dinucleotide motif from the K ODN significantly reduces immune activation. Incorporating multiple CpGs in a single K ODN increases immune stimulation. Preferably, the K ODN are at least 12 bases long. In addition, K ODN containing CpG motifs at the 5' end are the most stimulatory,  
15 although at least one base upstream of the CpG is required. More particularly, the most active K ODNs contain a thymidine immediately 5' from the CpG dinucleotide, and a TpT or a TpA in a position 3' from the CpG motif. Modifications which are greater than 2 base pairs from the CpG dinucleotide motif appear to have little effect on K ODN activity.

20 D type ODNs differ both in structure and activity from K type ODNs. The unique activities of D type ODNs are disclosed below (see section C). For example, as disclosed herein, D oligodeoxynucleotides stimulate the release of cytokines from cells of the immune system. In specific, non-limiting examples D type oligonucleotides stimulate the release or production of IP-10  
25 and IFN- $\alpha$  by monocytes and/or plasmacitoid dendritic cells and the release or production of IFN- $\gamma$  by NK cells. The stimulation of NK cells by D oligodeoxynucleotides can be either direct or indirect.

With regard to structure, in one embodiment, a CpG motif in a D type oligonucleotides has been described by Formula II:



wherein the central CpG motif is unmethylated, R is A or G (a purine), and Y is C or T (a pyrimidine). D-type oligonucleotides include an unmethylated CpG dinucleotide. Inversion, replacement or methylation of the CpG reduces or abrogates the activity of the D oligonucleotide.

In one embodiment, a D type ODN is at least about 16 nucleotides in length and includes a sequence represented by Formula III:



wherein the central CpG motif is unmethylated, Pu is a purine nucleotide, Py is a pyrimidine nucleotide, X and W are any nucleotide, M is any integer from 0 to 10, and N is any integer from 4 to 10.

The region  $Pu_1 Py_2 CpG Pu_3 Py_4$  is termed the CpG motif. The region  $X_1 X_2 X_3$  is termed the 5' flanking region, and the region  $X_4 X_5 X_6$  is termed the 3' flanking region. If nucleotides are included 5' of  $X_1 X_2 X_3$  in the D ODN these nucleotides are termed the 5' far flanking region. Nucleotides 3' of  $X_4 X_5 X_6$  in the D ODN are termed the 3' far flanking region.

In one specific non-limiting example,  $Py_2$  is a cytosine. In another specific, non-limiting example,  $Pu_3$  is a guanidine. In yet another specific, non-limiting example,  $Py_2$  is a thymidine and  $Pu_3$  is an adenine. In a further specific, non-limiting example,  $Pu_1$  is an adenine and  $Py_2$  is a tyrosine. In another specific, non-limiting example,  $Pu_3$  is an adenine and  $Py_4$  is a tyrosine.

In one specific not limiting example, N is from about 4 to about 8. In another specific, non-limiting example, N is about 6.

D-type CpG oligonucleotides can include modified nucleotides. Without being bound by theory, modified nucleotides can be included to increase the stability of a D-type oligonucleotide. Without being bound by theory, because phosphorothioate-modified nucleotides confer resistance to exonuclease digestion, the D ODN are "stabilized" by incorporating phosphorothioate-modified nucleotides. In one embodiment, the CpG dinucleotide motif and its immediate flanking regions include phosphodiester

rather than phosphorothioate nucleotides. In one specific non-limiting example, the sequence  $Pu_1 Py_2 CpG Pu_3 Py_4$  includes phosphodiester bases. In another specific, non-limiting example, all of the bases in the sequence  $Pu_1 Py_2 CpG Pu_3 Py_4$  are phosphodiester bases. In yet another specific, non-limiting example,  $X_1 X_2 X_3$  and  $X_4 X_5 X_6 (W)_M (G)_N$  include phosphodiester bases. In yet another specific, non-limiting example,  $X_1 X_2 X_3 Pu_1 Py_2 CpG Pu_3 Py_4 X_4 X_5 X_6 (W)_M (G)_N$  include phosphodiester bases. In further non-limiting examples the sequence  $X_1 X_2 X_3$  includes at most one or at most two phosphothioate bases and/or the sequence  $X_4 X_5 X_6$  includes at most one or at most two phosphothioate bases. In additional non-limiting examples,  $X_4 X_5 X_6 (W)_M (G)_N$  includes at least 1, at least 2, at least 3, at least 4, or at least 5 phosphothioate bases. Thus, a D type oligodeoxynucleotide can be a phosphorothioate/phosphodiester chimera.

As disclosed herein, any suitable modification can be used in the present invention to render the D oligodeoxynucleotide resistant to degradation in vivo (e.g., via an exo- or endo-nuclease). In one specific, non-limiting example, a modification that renders the oligodeoxynucleotide less susceptible to degradation is the inclusion of nontraditional bases such as inosine and quesine, as well as acetyl-, thio- and similarly modified forms of adenine, cytidine, guanine, thymine, and uridine. Other modified nucleotides include nonionic DNA analogs, such as alkyl or aryl phosphonates (i.e., the charged phosphonate oxygen is replaced with an alkyl or aryl group, as set forth in U.S. Patent No. 4,469,863), phosphodiester and alkylphosphotriesters (i.e., the charged oxygen moiety is alkylated, as set forth in U.S. Patent No. 5,023,243 and European Patent No. 0 092 574). Oligonucleotides containing a diol, such as tetraethyleneglycol or hexaethyleneglycol, at either or both termini, have also been shown to be more resistant to degradation. The D type oligodeoxynucleotides can also be modified to contain a secondary structure (e.g., stem loop structure). Without being bound by theory, it is believed that incorporation of a stem loop structure renders and oligodeoxynucleotide more effective.

In a further embodiment,  $Pu_1 Py_2$  and  $Pu_3 Py_4$  are self-complementary. In another embodiment,  $X_1 X_2 X_3$  and  $X_4 X_5 X_6$  are self complementary. In yet another embodiment  $X_1 X_2 X_3 Pu_1 Py_2$  and  $Pu_3 Py_4 X_4 X_5 X_6$  are self complementary.

5           Specific non-limiting examples of a D type oligonucleotide wherein  $Pu_1 Py_2$  and  $Pu_3 Py_4$  are self-complementary include, but are not limited to, ATCGAT, ACCGGT, ATCGAC, ACCGAT, GTCGAC, or GCCGGC. Without being bound by theory, the self-complementary base sequences can help to form a stem-loop structure with the CpG dinucleotide at the apex to facilitate  
10 immunostimulatory functions. Thus, in one specific, non-limiting example, D type oligonucleotides wherein  $Pu_1 Py_2$  and  $Pu_3 Py_4$  are self-complementary induce higher levels of IFN- $\gamma$  production from a cell of the immune system (see below). The self-complementary need not be limited to  $Pu_1 Py_2$  and  $Pu_3 Py_4$ . Thus, in another embodiment, additional bases on each side of the three  
15 bases on each side of the CpG-containing hexamer form a self-complementary sequence (see above).

One specific, non-limiting example of a sequence wherein  $Pu_1 Py_2$  and  $Pu_3 Py_4$  are self-complementary but wherein the far-flanking sequences are not self-complementary is

20           GGTGCATCGATACAGGGGGG (ODN D 113, SEQ ID NO:11).

This oligodeoxynucleotide has a far flanking region that is not self complementary and induces high levels of IFN- $\gamma$  and IFN- $\alpha$ .

Another specific, non-limiting example of a D oligodeoxynucleotides is:

GGTGCGTCGATGCAGGGGGG (D28, SEQ ID NO:7).

25           This oligodeoxynucleotide is of use for inducing production and/or release of cytokines from immune cells, although it lacks a self-complementary motif.

In one embodiment, the D type oligodeoxynucleotides disclosed herein are at least about 16 nucleotides in length. In a second embodiment, a D type  
30 oligodeoxynucleotide is at least about 18 nucleotides in length. In another

embodiment, a D type oligodeoxynucleotide is from about 16 nucleotides in length to about 100 nucleotides in length. In yet another embodiment, a D type oligodeoxynucleotide is from about 16 nucleotides in length to about 50 nucleotides in length. In a further embodiment, a D type oligodeoxynucleotide  
5 is from about 18 nucleotides in length to about 30 nucleotides in length.

In another embodiment, the oligodeoxynucleotide is at least 18 nucleotides in length, and at least two G's are included at the 5' end of the molecule, such that the oligodeoxynucleotide includes a sequence represented by Formula IV:

10



15

The D type oligodeoxynucleotide can include additional G's at the 5' end of the oligodeoxynucleotide. In one specific example, about 1 or about 2 G's are included at the 5' end of an oligodeoxynucleotide including a sequence as set forth as Formula IV.

Examples of a D type oligodeoxynucleotide include, but are not limited to, 5'XXTGCATCGATGCAGGGGGG 3' (SEQ ID NO: 12)

20

5'XXTGCACCGGTGCAGGGGGG3' (SEQ ID NO: 13),

5'XXTGCGTCGACGCAGGGGGG3' (SEQ ID NO: 14),

5'XXTGCGTCGATGCAGGGGGG3' (SEQ ID NO: 16),

5'XXTGCGCCGGCGCAGGGGGG3' (SEQ ID NO: 17),

5'XXTGCGCCGATGCAGGGGGG3' (SEQ ID NO: 18),

5'XXTGCATCGACGCAGGGGGG3' (SEQ ID NO: 19),

25

5'XXTGCGTCGGTGCAGGGGGG3' (SEQ ID NO: 20),

wherein X any base, or is no base at all. In one specific, non-limiting example, X is a G.

The oligodeoxynucleotides disclosed herein can be synthesized *de novo* using any of a number of procedures well known in the art. For



example, the oligodeoxynucleotides can be synthesized as set forth in U.S. Patent No. 6,194,388, which is herein incorporated by reference in its entirety. A D type oligodeoxynucleotide may be synthesized using, for example, the B-cyanoethyl phosphoramidite method or nucleoside H-phosphonate method.

- 5 These chemistries can be performed by a variety of automated oligonucleotide synthesizers available in the market. Alternatively, oligodeoxynucleotides can be prepared from existing nucleic acid sequences (e.g. genomic or cDNA) using known techniques, such as employing restriction enzymes, exonucleases or endonucleases, although this method is
- 10 less efficient than direct synthesis.

#### B. Delivery Complexes and Pharmaceutical Compositions

- In one embodiment, a D type oligodeoxynucleotide is included in delivery complex. The delivery complex can include the D type ODN and a
- 15 targeting means. Any suitable targeting means can be used. For example, a D type oligodeoxynucleotide can be associated with (e.g., ionically or covalently bound to, or encapsulated within) a targeting means (e.g., a molecule that results in higher affinity binding to a target cell, such as a B cell). A variety of coupling or cross-linking agents can be used to form the
- 20 delivery complex, such as protein A, carbodiimide, and N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP). Examples of an oligodeoxynucleotide delivery complexes include a D type oligodeoxynucleotide associated with a sterol (e.g., cholesterol), a lipid (e.g., a cationic lipid, anionic lipid, virosome or liposome), and a target cell specific binding agent (e.g., a ligand recognized
- 25 by target cell specific receptor). Without being bound by theory, the complex is sufficiently stable *in vivo* to prevent significant uncoupling prior to delivery to the target cell. In one embodiment, the delivery complex is cleavable such that the oligodeoxynucleotide is released in a functional form at the target cells.

- 30 In one embodiment, a pharmacological composition is provided that includes a D type oligonucleotide and a pharmacologically acceptable carrier.

Pharmacologically acceptable carriers (e.g., physiologically or pharmaceutically acceptable carriers) are well known in the art. A suitable pharmacological composition can be formulated to facilitate the use of a D type ODN *in vivo* and/or *ex vivo*. Such a composition can be suitable for  
5 delivery of the active ingredient to any suitable host, such as a patient for medical application, and can be manufactured in a manner that is itself known, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

10 Pharmacological compositions for use can be formulated in a conventional manner using one or more pharmacologically (e.g., physiologically or pharmaceutically) acceptable carriers comprising excipients, as well as optional auxiliaries that facilitate processing of the active  
15 compounds into preparations which can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen, and whether use will be an *in vivo* or an *ex vivo* use. For use *in vivo*, administration can be either systemic or local. In addition, one of skill in the art can readily select a suitable route of administration, including, but not  
20 limited to intravenous, intramuscular, intraperitoneal, transmucosal, subcutaneous, transdermal, transnasal, and oral administration.

Thus, for injection, the active ingredient can be formulated in aqueous solutions, preferably in physiologically compatible buffers. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art. For oral  
25 administration, the active ingredient can be combined with carriers suitable for inclusion into tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like. For administration by inhalation, the active ingredient is conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebuliser, with the use of a suitable  
30 propellant. The active ingredient can be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Similarly, D type oligodeoxynucleotides can be formulated for intratracheal or

for inhalation. Such compositions can take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and can contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Other pharmacological excipients are known in the art.

5           C.     Method of Inducing an Immune Response

A method is disclosed herein for stimulating a cell of the immune system. The method includes contacting the cell with an effective amount of a D type oligodeoxynucleotide disclosed herein, thereby stimulating the cell (see also PCT Application Nos. WO0061151A3, WO9956755A1, 10 WO9840100A1, WO9818810A1, WO0122990A2; which are herein incorporated by reference in their entirety).

In contrast, D type oligodeoxynucleotides have different immunostimulatory activities. As disclosed herein, administration of a D type oligodeoxynucleotide activates monocytes and/or natural killer cells, and 15 induces the maturation of dendritic cells. Furthermore, a D type oligodeoxynucleotide can be used to increase the production of cytokines (for example IP-10, IFN- $\alpha$  or IFN- $\gamma$ ) by a cell of the immune system.

Administration of the D type oligodeoxynucleotide can be by any suitable method. For example, the ODN can be administered *in vivo* or *ex vivo*. 20

Thus, in one embodiment, a method is also disclosed herein for producing an immune response in a subject. The subject can be any mammal, particularly a primate, such as a human. The method includes administering a D type oligodeoxynucleotide to the subject, thereby inducing 25 the immune response. In yet one embodiment, the immune response includes induction of the maturation of a dendritic cell or the activation of a natural killer cell and/or a monocyte. In a further embodiment, the immune response includes the production of a cytokine, such as, for example, IL-10, IP-10, IFN- $\alpha$  or IFN- $\gamma$ .

30           In one embodiment, a method is provided for inducing an immune response in a subject wherein the method includes contacting a monocyte or

a dendritic cell precursor *in vitro* with a D type oligodeoxynucleotide to produce an activated antigen presenting cell. The monocytes or dendritic cell precursors can be contacted with the D type oligodeoxynucleotides in the presence of or in the absence of antigen. The activated antigen presenting  
5 cell is then administered to the subject to induce an immune response.

In another embodiment, a method is provided herein for inducing an immune response in a subject that includes contacting a monocyte or a dendritic cell precursor *in vitro* with a D type oligodeoxynucleotide to produce an activated antigen presenting cell. The monocytes or dendritic cell  
10 precursors can be contacted with the D type oligodeoxynucleotides in the presence of or in the absence of antigen. Lymphocytes or natural killer are then contacted with the activated antigen presenting cells *in vitro*, or with cytokines secreted by the activated antigen presenting cells *in vitro*, to produce activated lymphocytes or activated natural killer cells. The activated  
15 lymphocytes or natural killer cells are administered to the subject to induce the immune response.

In order to induce an immune response, a D type oligodeoxynucleotide is administered either alone or in conjunction with another molecule. Co-administration includes administering the molecule and the D type  
20 oligodeoxynucleotide at the same time, or sequentially. The other molecule can be any other agent, such as a protein, an antigenic epitope, a hydrocarbon, lipid, mitogen, an anti-infectious agent (such as antiviral, antifungal, or anti-bacterial agent) or a vaccine (such as a live, attenuated, or heat-killed vaccine).

Without being bound by theory, when administered to a subject, it is believed that the ODNs initially act on antigen presenting cells (e.g., B cells, macrophages and dendritic cells). These cells then release cytokines, which activate natural killer (NK) cells. The activation of natural killer cells may be direct (e.g. through contact of the NK cell with a D type ODN) or indirect (e.g.  
30 by activating the secretion of cytokines, which then activate the natural killer cells). Either a cell-mediated or humoral immune response then occurs in the host.

As disclosed herein, D type oligodeoxynucleotides are a unique type of CpG containing oligodeoxynucleotides that have specific effects on the cells of the immune system. For several examples, D type oligonucleotides activate natural killer cells and monocytes, and induce the maturation of dendritic cells. In other examples, D type oligodeoxynucleotides increase production of cytokines such as IP-10, IL-10, IFN $\alpha$  and IFN- $\gamma$ . These effects are different from the effects of K type oligodeoxynucleotides. As previously described, K type oligodeoxynucleotides support B cell proliferation, and induce the production of IL-6. Thus, the elucidation of the immunostimulatory effects of D type oligodeoxynucleotides allows for customizing or tailoring of the type of immune obtained by administration of oligodeoxynucleotides. For example, a K type oligodeoxynucleotide can be utilized when production of IL-6 is desired, whereas D type oligodeoxynucleotides can be used when production of IFN- $\gamma$  is desired.

In one embodiment, a D type oligodeoxynucleotide is administered to a subject, such as a subject that has an autoimmune disease. Specific, non-limiting examples of autoimmune diseases include, but are not limited to diabetes, rheumatoid arthritis, lupus erythematosus, and multiple sclerosis. In one embodiment, the subject has cancer.

Also disclosed herein are methods of use to treat, prevent, or ameliorate an allergic reaction in a subject. An allergy refers to an acquired hypersensitivity to a substance (i.e., an allergen). Allergic conditions include eczema, allergic rhinitis or coryza, hay fever, bronchial asthma, urticaria (hives), food allergies, and other atopic conditions. The list of allergens is extensive and includes pollens, insect venoms, animal dander, dust, fungal spores, and drugs (e.g., penicillin). Examples of natural, animal, and plant allergens can be found in International Patent Application WO 98/18810. In one embodiment a D-type oligodeoxynucleotide administered to a subject to treat an allergic condition such as allergic asthma. In another embodiment, the D type oligodeoxynucleotide is administered in combination with any suitable anti-allergenic agent. Suitable anti-allergenic agents include those substances given in treatment of the various allergic conditions described

above, examples of which can be found in the Physicians' Desk Reference (1998).

In another embodiment, a D type oligodeoxynucleotide is administered to a subject that has a neoplasm. The D-type oligodeoxynucleotide is  
5 administered either alone or in combination with any suitable anti-neoplastic agent, such as a chemotherapeutic agent or radiation. Suitable neoplasms include, but are not limited to, solid tumors such as cancers of the brain, lung (e.g., small cell and non-small cell), ovary, breast, prostate, and colon, as well  
10 that the D type oligodeoxynucleotide increases the immune response to the neoplasm, and thus is involved in the reduction of tumor burden.

In a further embodiment, a method is provided to enhance the efficacy of any suitable vaccine. Suitable vaccines include those directed against Leishmania, Hepatitis A, B, and C, examples of which can be found in the  
15 Physicians' Desk Reference (1998), and DNA vaccines directed against, for example, malaria. (See generally Klinman et al., 17 *Vaccine* 17: 19, 1999; McCluskie and Davis, *J. Immun.* 161:4463, 1998).

D type oligodeoxynucleotides can be used to treat, prevent, or ameliorate any condition associated with an infectious agent. The D type  
20 oligodeoxynucleotide can be administered to a subject infected with the infectious agent alone or in combination with any suitable anti-infectious agent, such as an antiviral, anti-fungal or anti-bacterial agent (see Physicians' Desk Reference, 1998). Specific, non-limiting examples of infectious agents conditions associated with infectious agents are tularemia, francisella,  
25 schistosomiasis, tuberculosis, malaria, and leishmaniasis. Examples of infectious agents are viruses, bacteria, fungi, and other organisms (e.g., protists) can be found in International Patent Application WO 98/18810.

The D type oligodeoxynucleotides disclosed herein can also be used with any suitable antisense therapy. Suitable antisense agents are those that  
30 specifically bind either with a target DNA or a target RNA and inhibit expression of the target sequence (see Lonnberg et al., *Ann. Med.* 28: 511,

1996; Alama et al., *Pharmacol. Res.* 36: 171, 1997; Scanlon et al., *FASEB J.* 9: 1288, 1995; Oberbauer, 109 *Wien Klin Wochenschr* 109: 40, 1997).

The present invention is further described in the following examples.

- 5 These examples are intended only to illustrate the invention and are not intended to limit the scope of the invention in any way.

## EXAMPLES

### Example 1

#### Cytokine production

- 10 The oligodeoxynucleotides disclosed herein can be used to produce an immune response. In one embodiment, an immune response can be measured by cytokine production. As shown herein, K type oligodeoxynucleotides can be used to increase the production of the cytokines IL-6 and induce cell proliferation. D type oligodeoxynucleotides induce the  
15 production of IFN- $\gamma$ .

- Human peripheral blood mononuclear cells (PBMC) were isolated, as described elsewhere (Ballas et al., *J. Allergy Clin. Immunol.* 85: 453, 1990; Ballas and Rasmussen, *J. Immunol.* 150: 1039, 1990; Ballas and Rasmussen, *J. Immunol.* 150: 17, 1993). Oligodeoxynucleotides (ODNs) were synthesized  
20 on a DNA synthesizer (Applied Biosystems Inc., Foster City, CA), as described elsewhere (Beacage and Caruthers, *Tetrahedron Letters* 22: 1859, 1981). In some ODNs, the normal DNA backbone phosphodiesterase linkages were replaced with phosphorothioate linkages, as described elsewhere (Agrawal et al., *Proc. Natl. Acad. Sci. USA* 94: 2620, 1997;  
25 Agrawal *TIB TECH* 14: 376, 1996). To reduce degradation of the ODNs, those that did not have an entire phosphorothioate backbone contained phosphorothioate linkages at the 5' and 3' ends. Cells were incubated for approximately 72 hrs with the various ODNs. IL-6 and TNF- $\gamma$  levels were determined by ELISA using anti-IL-6 and anti-TNF- $\gamma$  antibodies, as described  
30 elsewhere (Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold

Spring Harbor Laboratory Press, New York, 1989). Cell proliferation was determined by [<sup>3</sup>H] thymidine incorporation, as described elsewhere (Liang et al., J Clin. Invest. 98:1121, 1996).

IL-6 levels and cell proliferation are set forth in Table 1: Induction of a Humoral Immune Response In Vitro. These data demonstrate that a sequence containing 5' N<sub>1</sub>N<sub>2</sub>N<sub>3</sub>T-CpG-WN<sub>4</sub>N<sub>5</sub>N<sub>6</sub> 3', wherein the central CpG motif is unmethylated, W is A or T, and N<sub>1</sub>, N<sub>2</sub>, N<sub>3</sub>, N<sub>4</sub>, N<sub>5</sub>, and N<sub>6</sub> are any nucleotides, (K type oligodeoxynucleotides) induce a the production of IL-6 and a humoral immune response. However, D type oligodeoxynucleotides induce only low levels of IL-6 production.

In addition, maximum induction was observed for K type ODNs that contained a phosphorothioate backbone. IFN- $\gamma$  levels and cell proliferation are set forth in Table 1. These data demonstrate that D type oligonucleotides increase production of IFN- $\gamma$ . Maximum induction occurred with ODNs containing phosphodiesterase linkages.

**Table 1**

Underlined bases are phosphodiester. \* indicates methylated CG. Bold indicates self-complementary sequences. Sequence identifier is noted below the nucleic acid sequence. For each heading (IFN-g, IL-6, IgM, PROLIF (proliferation)) first column is average and second column is standard deviation.

ODN	SEQUENCE	IFN-g*		IL-6*		IgM*		PROLIF*	
media	(None)	0.9	0.8	0.7	1	0.5	0.5	0.9	0.6
<b>D ODN</b>									
DV104	GGTGCATCGATGCAGGGGGG	47.7	64.7	30.8	31	10.2	12.3	2.9	1.7
	(SEQ ID NO:1)								
DV19	GGTGCATCGATGCAGGGGGG	44.0	71.8	12.5	17	9.6	21.0	3.1	3.1
	(SEQ ID NO:1)								
DV29	GGTGCACCGGTGCAGGGGGG	35.9	38.9	3.8	4	2.5	2.9	1.7	1.2
	(SEQ ID NO:2)								



	DV35	GGTGCATCGATGCAGGGGGG	33.3	63.6	28.0	29	5.7	8.4	2.0	1.1
		(SEQ ID NO:1)								
	DV28	GGTGCCTCGATGCAGGGGGG	32.2	45.3	13.8	23	9.0	14.3	2.8	2.0
		(SEQ ID NO:7)								
5	DV106	GGTGTGTCTCGATGCAGGGGGG	29.9	71.4	7.5	6	32.2	94.5	2.9	1.8
		(SEQ ID NO:26)								
	DV116	TGCATCGATGCAGGGGGG	25.1	28.9	28.3	25	6.2	3.3	2.6	1.6
		(SEQ ID NO:12)								
10	DV113	GGTGCATCGATACAGGGGGG	21.1	15.3	144.8	255	4.0	4.2	4.7	5.6
		(SEQ ID NO:11)								
	DV34	GGTGCATCGATGCAGGGGGG	16.2	19.1	3.6	6	2.4	2.6	3.2	4.5
		(SEQ ID NO:1)								
	DV102	GGTGCATCGTTGCAGGGGGG	15.8	39.4	6.6	7	24.0	58.0	2.1	1.1
		(SEQ ID NO:30)								
15	DV32	GGTGCCTCGACGCAGGGGGG	14.5	18.0	2.7	5	1.4	2.1	2.8	0.9
		(SEQ ID NO:31)								
	DV117	GGTCTCATCGATGCACGGGGG	13.4	14.6	25.8	23	19.6	22.8	6.2	2.7
		(SEQ ID NO:32)								
20	DV37	GGTGCATCGATGCAGGGGGG	13.0	19.6	13.3	21	38.0	33.0	4.8	3.6
		(SEQ ID NO:1)								
	DV25	GGTGCATCGATGCAGGGGGG	12.2	7.9	2.0	3	2.1	2.7	0.9	0.7
		(SEQ ID NO:1)								
	DV30	GGTGCATCGACGCAGGGGGG	11.3	8.1	2.4	3	6.4	6.9	2.4	1.2
		(SEQ ID NO:35)								
25	dv120	GGTGCATCGATAGCGGGGGG	8.7	11.5	1.0	1	3.5	1.1	1.9	1.0
		(SEQ ID NO:36)								
	DV27	GGTGCAACGATGCAGGGGGG	8.5	7.3	2.9	4	3.3	4.9	1.9	1.5
		(SEQ ID NO:37)								
30	dv119	CCTGCATCGATGCAGGGGGG	7.5	9.1	2.0	1	1.6	0.4	1.0	0.3
		(SEQ ID NO:38)								
	D142	GGTATATCGATATAGGGGGG	7.3	1.1	15.9	5	22.5	5.0	2.0	1.0

(SEQ ID NO:39)

d143	GGTGGATCGATCCAGGGGGG	6.4	4.5	16.0	8	47.0	12.0	2.0	1.0
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(SEQ ID NO:40)

5 **D CONTROLS**

dv17	GGTGCAACGTTGCAGGGGGG	2.3	1.5	5.0	4	8.0	2.0	4.0	2.0
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(SEQ ID NO:41)

DV78	GGTGCATCGATAGAGGGGGG	0.8	0.5	2.0	4	4.2	3.0	1.4	0.9
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(SEQ ID NO:42)

10 DV96	GGTGCATCGTAGCAGGGGGG	1.1	0.7	1.5	2	1.1	2.9	1.2	0.3
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(SEQ ID NO:43)

DV95	GGTGGTTCGATGCAGGGGGG	2.1	2.2	2.5	3	1.2	1.8	1.2	0.7
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(SEQ ID NO:44)

DV93	GGTGCATCGATGCAGGGGGG	0.9	1.3	11.9	26	2.7	3.5	1.5	0.6
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(SEQ ID NO:1)

15

DV92	GGTGCAACCGGTGCAAAAAA	0.4	0.7	2.0	3	0.8	1.2	1.4	0.8
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(SEQ ID NO:46)

DV81	GGTGCATCGATAGAGGGG	0.8	1.1	1.2	2	1.2	1.6	1.4	0.6
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(SEQ ID NO:47)

20 DV77	GGTGCATCGATGCAGGGGGG	1.0	1.6	9.5	13	82.1	181.9	6.5	12.2
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(SEQ ID NO:1)

DV76	GGTGCATCGATGCAAAAAA	1.6	1.5	1.3	2	1.2	1.5	1.7	0.4
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(SEQ ID NO:49)

DV71	GGGGTCGACAGGG	1.4	2.2	1.8	2	0.7	0.5	1.2	1.0
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(SEQ ID NO:50)

25

DV49	GGTGCAATAATGCAGGGGGG	1.5	1.2	1.3	2	1.0	1.0	1.2	1.0
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(SEQ ID NO:51)

DV48	GGTGCATCAATGCAGGGGGG	1.0	0.5	1.2	2	1.0	1.0	1.2	1.0
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(SEQ ID NO:52)

30 DV47	GGTGCAATTGATGCAGGGGGG	1.2	0.5	1.5	2	1.0	1.0	1.2	1.0
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(SEQ ID NO:53)

	DV45	GGTGCATC*GATGCAGGGGGG	3.4	2.9	7.0	14	0.8	1.5	1.3	0.3
		(SEQ ID NO:54)								
	DV26	GGTGCATGCATGCAGGGGGG	7.6	15.3	0.0	0	0.6	1.0	1.3	1.4
		(SEQ ID NO:55)								
5	DV20	GGTGCATGCATGCAGGGGGG	2.3	4.2	2.1	2	1.0	1.8	1.1	0.8
		(SEQ ID NO:55)								
	DV122	GGTGCATTGATGCAGGGGGG	0.9	1.5	1.3	1	2.0	0.4	0.7	0.3
		(SEQ ID NO:53)								
	DV114	GGTGCACTGGTGCAGGGGGG	1.8	2.0	1.8	2	1.2	1.2	1.0	0.6
10		(SEQ ID NO:58)								
	DV111	GGTGTATCGATGCAAAAGGG	5.8	5.1	12.3	13	4.8	7.6	4.4	8.0
		(SEQ ID NO:59)								
	DV108	GGTGCCCCGTTGCAGGGGGG	1.2	1.4	1.7	3	1.0	1.5	1.0	0.2
		(SEQ ID NO:60)								
15	DV107	GGTGCAACGGGGCAGGGGGG	2.5	3.3	7.7	15	0.4	0.6	1.1	0.4
		(SEQ ID NO:61)								
	DV105	AATGCATCGATGCAAAAAA	1.1	1.8	2.4	3	1.2	1.5	1.0	0.4
		(SEQ ID NO:62)								
	DV103	GGTGCAACCGTGGCAGGGGGG	9.4	16.8	8.7	12	5.4	8.4	1.7	1.9
20		(SEQ ID NO:63)								
	DV100	GGTGCATCGAAGCAGGGGGG	3.4	3.5	7.2	6	21.0	24.0	9.1	5.9
		(SEQ ID NO:64)								
	d79	GGTGGATCGATGCAGGGGGG	0.6	0.5	1.0	1	23.0	2.0		
		(SEQ ID NO:65)								
25	d145	GGTGACGCGTGCAGGGGGG	4.8	0.8	2.5	4	16.8	3.0		
		(SEQ ID NO:5)								
	d144	GGTGCATGTATGCAGGGGGG	0.0	0.0	0.6	1	1.3	2.0		
		(SEQ ID NO:67)								
	AA20	GGGGGATCGATGGGGG	2.7	5.1	3.1	3	2.2	2.5	1.6	1.2
30		(SEQ ID NO:68)								
	AA3M	GGGGGAAGCTTCGGGG	2.2	3.2	1.1	2	0.4	0.8	1.2	1.0

(SEQ ID NO:69)

**K ODN**

5	K22	CTCGAGCGTTCTC	9.3	1.8	43.3	20	131.8	94.0	18.3	3.3
		(SEQ ID NO:70)								
	DV84	ACTCTCGAGCGTTCTA	1.1	1.6	42.6	91	79.7	105.3	18.5	25.8
		(SEQ ID NO:71)								
	K21	TCTCGAGCGTTCTC	7.2	2.8	39.6	17	237.6	194.2	34.6	9.3
10		(SEQ ID NO:72)								
	K82	ACTCTGGAGCGTTCTC	1.9	0.1	38.5	5	25.5	16.8	10.7	0.6
		(SEQ ID NO:73)								
	K30	TGCAGCGTTCTC	9.5	10.5	30.8	31	253.1	256.3	7.0	6.9
		(SEQ ID NO:74)								
15	k31	TCGAGGCTTCTC	22.1	26.3	30.7	18	290.3	150.6	12.9	9.0
		(SEQ ID NO:75)								
	K39	GTCGGCGTTGAC	7.0	11.0	27.3	22	100.3	137.9	16.1	6.3
		(SEQ ID NO:76)								
	K16	TCGACTCTCGAGCGTTCTC	3.1	3.1	25.2	21	72.7	79.5	22.3	20.6
20		(SEQ ID NO:8)								
	K3	ATCGACTCTCGAGCGTTCTC	9.0	22.7	22.6	17	113.5	60.0	25.0	19.4
		(SEQ ID NO:3)								
	k23	TCGAGCGTTCTC	5.2	8.3	21.9	28	111.3	145.4	28.3	16.0
		(SEQ ID NO:79)								
25	DV110	TCGAGGCTTCTC	2.2	2.2	21.8	25	70.3	142.8	19.6	19.8
		(SEQ ID NO:75)								
	K40	GTCGGCGTCGAC	11.3	14.3	20.7	11	127.1	222.2	11.0	5.0
		(SEQ ID NO:81)								
	DV101	CTCGAGCGTTCT	2.6	2.2	20.0	26	28.0	34.3	25.3	28.3
30		(SEQ ID NO:10)								
	DV89	ACTCTTTCGTTCTC	1.6	1.6	19.7	23	169.1	390.3	6.7	7.2

		(SEQ ID NO:83)							
	K34	GTCGACGTTGAC	4.3	5.3	17.2	8	265.8	273.4	11.3 6.7
		(SEQ ID NO:84)							
	DV86	ACTCTCGAGCGTTCTC	0.8	1.6	16.9	37	36.0	40.7	5.1 3.0
5		(SEQ ID NO:9)							
	K83	ACTCTCGAGGGTTCTC	0.0	0.0	16.5	5	16.9	9.7	6.9 0.7
		(SEQ ID NO:86)							
	K19	ACTCTCGAGCGTTCTC	4.8	7.9	14.8	16	151.0	223.6	23.4 16.1
		(SEQ ID NO:9)							
10	DV88	ACTCTCGAGCGTTCTCAAAA	1.9	3.0	14.5	14	49.7	55.0	10.2 11.5
		(SEQ ID NO:88)							
	DV85	CATCTCGAGCGTTCTC	0.9	1.5	12.0	11	82.0	126.8	12.2 13.0
		(SEQ ID NO:89)							
	K73	GTCGTCGATGAC	4.5	5.2	11.7	7	99.2	110.7	17.7 13.2
15		(SEQ ID NO:90)							
	DV109	TCGAGCGTTCT	2.2	2.2	11.3	9	28.4	46.4	14.5 12.6
		(SEQ ID NO:4)							
	D123	TCGTTGTTCTC	3.3	1.6	9.6	12	50.9	72.3	39.3 30.5
		(SEQ ID NO:92)							
20	D124	TCGTTGTTCTC	3.0	2.0	7.3	6	63.2	88.6	34.1 28.1
		(SEQ ID NO:93)							
	K46	GTCGACGCTGAC	3.5	5.1	5.2	2	21.9	15.1	8.7 5.0
		(SEQ ID NO:94)							
	D139	TCGATGCTTCTC	2.2	1.4	5.1	4	97.8	157.5	39.1 27.9
25		(SEQ ID NO:95)							
	D137	TCGCCGCTTCTC	1.4	0.5	4.3	2	78.7	107.6	31.6 22.3
		(SEQ ID NO:96)							
	K47	GTCGACGTCGAC	3.8	7.1	3.9	1	14.1	6.2	5.8 2.3
		(SEQ ID NO:54)							
30	K72	GTCATCGATGCA	1.8	2.3	3.7	3	95.6	79.3	11.0 5.4
		(SEQ ID NO:98)							

	DV90	ACTCTTTTCGATCTC	1.6	2.0	3.6	4	19.2	24.8	4.6	5.7
		(SEQ ID NO:99)								
	K37	GTCAGCGTCGAC	10.8	19.5	3.4	1	52.4	63.0	6.4	2.2
		(SEQ ID NO:100)								
5	k25	TCGAGCGTTCT	3.6	3.7	2.9	4	17.9	24.8		
		(SEQ ID NO:4)								
	D127	TGGAGCGTTCTC	2.3	1.6	2.8	3	89.7	136.4	21.4	11.9
		(SEQ ID NO:102)								
10	D138	TGCTGCGTTCTC	0.7	0.7	2.1	1	69.0	105.5	18.4	12.1
		(SEQ ID NO:103)								
	D125	TTGAGCGTACTC	3.3	3.2	2.0	1	35.1	53.8	7.8	5.2
		(SEQ ID NO:104)								
	D134	TGCTTCGAGCTC	1.8	0.8	1.8	1	17.7	24.8	9.1	5.7
		(SEQ ID NO:105)								
15	D136	TGCACCGTTCTC	1.7	0.2	1.7	1	69.2	106.5	12.1	7.9
		(SEQ ID NO:106)								
	<b>CONTROL K ODN</b>									
20	DV89	ACTCTTTTCGTTCTC	1.6	1.6	19.7	23	169.1	419.7	6.7	7.2
		(SEQ ID NO:83)								
	d112	TGCAGGCTTCTC	16.0	28	20.4	32.6				
		(SEQ ID NO:6)								
	DV112	TTGAGTGTTCTC	2.1	2.7	16.0	28	20.4	32.6	9.8	10.8
		(SEQ ID NO:109)								
25	DV112	TTGAGTGTTCTC	2.1	2.7	16.0	28	20.4	32.6	9.8	10.8
		(SEQ ID NO:109)								
	K41	GTCGGCGCTGAC	5.7	7.4	13.3	8	29.1	24.7	8.8	5.7
		(SEQ ID NO:120)								
	DV109	TCGAGCGTTCT	3.4	4.0	11.3	9	28.4	46.4	14.5	12.6
30		(SEQ ID NO:4)								
	k10	ATGCACTCTGCAGGCTTCTC	2.7	4.5	6.1	7	27.3	28.8	5.7	5.4

		(SEQ ID NO:119)							
	K38	GTCAGCGCTGAC	1.4	1.9	4.5	3	4.5	1.3	2.2 1.1
		(SEQ ID NO:118)							
	k29	TCGAGCG	19.7	25.9	4.2	5			2.8 3.6
5		(SEQ ID NO:112)							
	k26	TCGAGCGTTC	6.6	4.9	4.1	1			22.3 24.4
		(SEQ ID NO:45)							
	k27	TCGAGCGTT	4.6	3.4	3.7	5			4.1 3.7
		(SEQ ID NO:108)							
10	K36	GTCAACGCTGAC	2.2	3.3	3.4	2	8.6	2.0	5.5 2.8
		(SEQ ID NO:107)							
	K35	GTCAACGTCGAC	21.8	41.9	3.3	2	15.9	13.9	5.0 2.8
		(SEQ ID NO:101)							
	K44	GTCGACGCCGAC	4.1	6.0	3.0	2	9.4	6.3	2.6 1.2
15		(SEQ ID NO:97)							
	k28	TCGAGCGT	21.5	26.2	2.9	3			6.7 9.1
		(SEQ ID NO:91)							
	AA19	GGGGGAACGTTGGGG	9.4	14.6	2.2	1	4.7	4.5	1.5 0.5
		(SEQ ID NO:87)							
20	D135	TGCAGCGAGCTC	1.4	0.5	1.8	2	4.3	6.6	3.4 1.5
		(SEQ ID NO:85)							
	D141	CCGAGGCTTCTC	1.3	0.4	1.7	2	53.7	99.0	12.8 9.0
		(SEQ ID NO:82)							
	D126	ACGAGGGTTCTC	2.6	0.8	1.7	1	75.5	107.5	15.2 8.7
25		(SEQ ID NO:80)							
	K42	GTCAACGCCGAC	2.6	3.1	1.7	1	5.7	5.3	4.3 3.7
		(SEQ ID NO:78)							
	D140	GCGAGGCTTCTC	1.2	0.5	1.5	1	56.7	85.4	10.9 7.0
		(SEQ ID NO:77)							
30	d121	ACTCTTGAGTGTCTC	3.6	6.2	1.0	1	10.2	18.5	17.3 44.5
		(SEQ ID NO:66)							

	K45	GTCGGCGCCGAC	5.7	6.6	0.4	1	18.3	22.1	10.1	4.3
		(SEQ ID NO:57)								
	K43	GTCAGCGCCGAC	2.1	1.6	0.3	1	6.9	8.6	5.1	5.7
		(SEQ ID NO:56)								
5	K24	CGAGCGTTCTC	4.9	5.1	0.1	0	0.7	1.0	1.0	0.1
		(SEQ ID NO:48)								

The foregoing data demonstrates the induction of an immune response in human cells, as exemplified by PBMC. Specifically, the results demonstrate that K type oligodeoxynucleotides induce IL-6, cell proliferation and a humoral response, while D type oligodeoxynucleotides induce the production of IFN- $\gamma$ .

## Example 2

### Production of IL-6

The following example demonstrates induction of an immune response, as measured by cytokine production, by K type oligodeoxynucleotides. Specifically, it is demonstrated that production of the cytokine IL-6 can be induced by K type oligodeoxynucleotides, and that B cells are stimulated by K type oligodeoxynucleotides.

A human B cell line (RPMI 8226) was maintained according to the manufacturers recommendations. ODNs were synthesized as described in Example 1. In some ODNs, the normal DNA phosphodiesterase linkages were replaced with phosphorothioate linkages, as described in Example 1. To reduce degradation of the ODNs, those that did not have an entire phosphorothioate backbone contained phosphorothioate linkages at the ends. The cells were incubated with various ODNs for 14 hrs. IL-6 production was determined by ELISA using anti-IL-6 antibodies, as described in Example 1.

IL-6 levels are set forth in Table 1. These data confirm that a sequence containing 5' N<sub>1</sub>N<sub>2</sub>N<sub>3</sub>T-CpG-WN<sub>4</sub>N<sub>5</sub>N<sub>6</sub> 3', which are linked by phosphorothioate bonds and wherein the central CpG motif is unmethylated,



W is A or T, and N<sub>1</sub>, N<sub>2</sub>, N<sub>3</sub>, N<sub>4</sub>, N<sub>5</sub>, and N<sub>6</sub> are any nucleotides, is desirable to induce a humoral immune response.

The foregoing data demonstrates the induction of an immune response in human cells by K type oligodeoxynucleotides, as exemplified by the human  
5 B cell line RPMI 8226, and as measured by production of the cytokine IL-6.

### Example 3

#### Exemplary Material and Methods

10 The following example delineates material and methods that were used to investigate the induction of an immune response by D-type ODNs. Exemplary D type ODNs are shown in Table 2 below.

**Table 2**

15 UNDERLINED= PHOSPHODIESTER; \*METHYLATED C

ODN		IFN $\gamma$	
		AVG	SD
20	Media	0.9	0.8
	DV104 GGTGCATCGATGCAGGGGGG (SEQ ID NO: 1)	47.7	64.7
	DV19 GGTGCATCGATGCAGGGGGG (SEQ ID NO: 1)	44.0	71.8
	DV29 GGTGCACCGGTGCAGGGGGG (SEQ ID NO: 2)	35.9	38.9
	DV35 GGTGCATCGATGCAGGGGGG (SEQ ID NO: 1)	33.3	63.6
25	DV28 GGTGCGTCGATGCAGGGGGG (SEQ ID NO: 7)	32.2	45.3
	DV106 GGTGTGTCGATGCAGGGGGG (SEQ ID NO: 26)	29.9	71.4
	DV116 TGCATCGATGCAGGGGGG (SEQ ID NO: 12)	25.1	28.9
	DV113 GGTGCATCGATACAGGGGGG (SEQ ID NO: 11)	21.1	15.3
	DV34 GGTGCATCGATGCAGGGGGG (SEQ ID NO: 1)	16.2	19.1
30	DV102 GGTGCATCGTTGCAGGGGGG (SEQ ID NO: 30)	15.8	39.4
	DV32 GGTGCGTCGACGCAGGGGGG (SEQ ID NO: 31)	14.5	18.0
	DV117 GGTTCGATCGATGCACGGGGG (SEQ ID NO: 32)	13.4	14.6
	dv37 GGTGCATCGATGCAGGGGGG (SEQ ID NO: 1)	13.0	19.6
	DV25 GGTGCATCGATGCAGGGGGG (SEQ ID NO: 1)	12.2	7.9
35	DV30 GGTGCATCGACGCAGGGGGG (SEQ ID NO: 35)	11.3	8.1
	dv120 GGTGCATCGATAGCGGGGGG (SEQ ID NO: 36)	8.7	11.5
	DV27 GGTGCACCGATGCAGGGGGG (SEQ ID NO: 37)	8.5	7.3
	dv119 CCTGCATCGATGCAGGGGGG (SEQ ID NO: 38)	7.5	9.1
	D142 GGTATATCGATATAGGGGGG (SEQ ID NO: 39)	7.3	1.1
40	d143 GGTGGATCGATCCAGGGGGG (SEQ ID NO: 40)	6.4	4.5

Normal PBMC were obtained from the National Institutes of Health Department of Transfusion Medicine (Bethesda, MD). The human myeloma cell line RPMI 8226 (CCL-155; American Type Culture Collection, Manassas, VA) and the NK-92 human NK cell line (a kind gift of Dr. J. Ortaldo, National Cancer Institute, Frederick, MD) were grown in RPMI 1640 supplemented with 10% FCS, penicillin/streptomycin, L-glutamine, HEPES, sodium pyruvate, and 2-ME in a 5% CO<sub>2</sub> in-air incubator. Medium for NK-92 cells was supplemented with IL-2 (200 IU/ml; R&D Systems, Minneapolis, MN) and IL-15 (15 ng/ml; Endogen, Boston, MA).

Oligodeoxynucleotides  
ODN were synthesized at the Center for Biologics Evaluation and Research core facility. All had <0.1 endotoxin U/ml endotoxin at ODN concentrations of 1 mg/ml.

Antibodies  
Abs against human IFN- $\gamma$  (Endogen), IL-6 (R&D Systems), and IgM (Se-rotec, Oxford, U.K.) were used for ELISA and enzyme-linked immunospot (ELISPOT) assays. FITC- and/or CyChrome-labeled Abs against human CD3, CD4, CD14, CD11c, CD16, CD56, CD83, HLA-DR, IL-6, and IFN- $\gamma$  were obtained from BD PharMingen (San Diego, CA) or BD Bio-sciences (San Jose, CA) and used as recommended by the manufacturer. Neutralizing Abs to IL-12 were obtained from R&D Systems, and Abs to IL-18 were kindly provided by Dr. Howard Young (National Cancer Institute).

Mononuclear cell preparation  
Mononuclear cells were separated by density gradient centrifugation over Ficoll-Hypaque as described (17). Cells were washed three times and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS for 72 h at 5  $\times$  10<sup>5</sup> cells/well in the presence of 1–3 mM ODN.

ELISA and ELISPOT assays  
Ninety-six-well microtiter plates (Millipore, Bedford, MA) were coated with anti-cytokine Ab or anti-IgM and blocked with PBS-5% BSA (17,18).

Cytokines and Ig in culture sups or secreted by individual cells were detected colorimetrically using biotin-labeled Abs followed by phosphatase conjugated avidin and then phosphatase-specific colorimetric substrate. Standard curves were generated to quantitate ELISA results using known amounts of recombinant cytokine or purified IgM. The detection limit of the assays was: 6 pg/ml for IFN-g, 20 pg/ml for IL-6, and 10 ng/ml for IgM. Stimulation index was calculated by the formula: (value for stimulated cells 2 background)/(value for unstimulated cells 2 background). In cases where cytokine/Ig production was below assay sensitivity, the lower limit of detection was used to calculate the stimulation indices. All assays were performed in triplicate.

#### Proliferation assays

A total of 10<sup>5</sup> PBMC/well were incubated with 3 mM of ODN for 68 h, pulsed with 1 mCi of [<sup>3</sup>H]thymidine, and then harvested 4 h later. The proliferation index represents the fold difference between stimulated and unstimulated cells. All assays were performed in triplicate.

#### Intracellular cytokine staining and flow cytometry

PBMC were cultured for 8 h (K type) or 24 h (D type) with 3 mM of various ODN. Brefeldin A (20 mg/ml) was added to the cultures after 2 or 12 h, respectively. Cells were harvested with warm PBS-0.02% EDTA and washed. PBMC (1.3 × 10<sup>6</sup> /sample) were fixed and permeabilized using the Fix & Perm cell permeabilization kit (Caltag, Burlingame, CA) as recommended by the manufacturer. Cells were then stained with PE-conjugated anti-IL-6 or anti-IFN γ plus specified FITC- or Cy Chrome-conjugated Abs against cell surface markers for 30 min in darkness. After labeling, the cells were washed twice, and 40,000 events per sample were analyzed by FAC-Scan flow cytometry (BD Biosciences). Cell Quest software (BD Biosciences) was used for data analysis.

### Statistical analysis

Statistically significant differences were determined using a two-tail non-parametric Mann-Whitney U test and nonparametric ANOVA.

5

### **Example 4**

#### **Response of Human Peripheral Blood Mononuclear Cells (PBMC) to CpG ODN**

Response of human PBMC to CpG ODN Novel ODN were studied for their ability to stimulate human PBMC to proliferate and/or secrete Ig or cytokines. As shown in Fig.1, two structurally distinct ODN classes were identified that stimulated PBMC from .95% of the donors. Those of the K type stimulated significantly greater cell proliferation (  $p < 0.0001$ ) and induced higher levels of IL-6 (240 vs 85 pg/ml;  $p < 0.01$ ) and IgM(695 vs 20 ng/ml;  $p < 0.0001$ ) than D ODN. In contrast, D ODN were stronger inducers of IFN-g (70 vs 13 pg/ml;  $p < 0.05$ ). Fig. 1 illustrates the response of PBMC to K and D ODN.

#### *Type D ODN*

Modifications were introduced in various regions of D ODN to identify the critical sequences and structures that account for the ability of these ODN to induce IFN- $\gamma$ . To standardize results from the large number of subjects and experiments included in the analysis, the magnitude of each response is presented as fold increase over cells from the same subject incubated in medium alone. The general magnitude of these responses was comparable to that shown in Fig. 1.

Fig. 2 illustrates the parameters governing D ODN induced immune activation. All D-type ODNs contain an unmethylated CpG dinucleotide (Fig. 2). Inversion, replacement, or methylation of the CpG reduces or abrogates reactivity (Fig. 2A, line 1 vs lines 2–6, and line 7 vs line 8;  $p < 0.0001$ ). In addition, the results demonstrate that D ODN are stimulatory only if the CpG dinucleotide and its immediate flanking regions are

composed of phosphodiester (shown in gray) rather than phosphorothioate nucleotides (Fig. 2B, line 1 vs line 2;  $p < 0.001$ ). Because phosphorothioate-modified nucleotides confer resistance to exonuclease digestion, they are incorporated at the ends of the ODN to improve activity (Fig. 2B, lines 1 and 5 versus lines 3 and 4;  $p < 0.07$ ). Unless otherwise stated, all D ODN studied were phosphorothioate/phosphodiester chimeras.

As shown in Fig. 2, the level of immune stimulation induced by D ODN was demonstrated to be influenced by the bases flanking the CpG dinucleotide. Self-complementary hexamers consisting of a Pu Py CG Pu Py were the most active, as exemplified by ATCGAT and ACCGGT (Fig. 2C, lines 1 and 2). Substituting a Pu for a Py, or vice versa, significantly reduced or eliminated ODN activity (circled nucleotides in Fig. 2C, lines 1 and 2 vs lines 6–13;  $p < 0.0001$ ). By comparison, hexamers that maintained the PuPyCGPuPy sequence, but are non self-complementary induced lower levels of IFN-g production (Fig. 2C, lines 1 and 2 vs lines 3–5;  $p < 0.001$ ). Sequential deletion experiments showed that the minimum length of an active D ODN is 16 bp (Fig. 2D;  $p < 0.01$ ). This finding suggested that sequences outside the central hexamer might influence D ODN activity.

Indeed, stimulation was maximal when the three bases on each side of the CpG-containing hexamer formed a self-complementary sequence (Fig. 2E, lines 1 and 2 versus lines 3 and 4;  $p < 0.0001$ ). Without being bound by theory, computer modeling of D ODN suggested that these self-complementary base sequences help form a stem-loop structure with the CpG dinucleotide at the apex at 37°C. The ends of the ODN also contribute to its activity, with the inclusion of more than four Gs at the 3' end significantly increased function (Fig. 2F, lines 1–3 versus lines 4 and 5;  $p < 0.001$ ). Thus, modifications in any of the three areas (the central hexamer, the region flanking the hexamer, or the poly G tail) can be used to influence ODN activity.

**Type K ODN**

K ODN trigger cell proliferation and the secretion of IgM and IL-6, but little IFN-g( see Table 1 and Fig. 1). These ODN have a phosphorothioate backbone and at least one unmethylated CpG dinucleotide.

5

**Table 5****Rules Governing K ODN Induced Immune Activation**

		Stimulation index					
		IL-6		Proliferation		IgM	
Assay Subject #:		<u>1</u>	<u>2</u>	<u>1</u>	<u>2</u>	<u>1</u>	<u>2</u>
10	A: Multiple CpGs induce more stimulation						
	1 ATCGACTCTCGAGCGTTCTC (SEQ ID NO: 3)	50	57	13	30	138	71
	2 TCGAGCGTTCTC (SEQ ID NO: 79)	35	40	15	37	19	79
15	3 TCGAGGCTTCTC (SEQ ID NO: 75)	28	12	8	25	8	22
	4 TGCAGGCTTCTC (SEQ ID NO: 6)	1	0	5	7	5	4
	B: Minimum size of stimulatory "K" ODN						
20	1 TCGACTCTCGAGCGTTCTC (SEQ ID NO: 8)	20	23	37	72	>100	100
	2 ACTCTCGAGCGTTCTC (SEQ ID NO: 9)	20	18	30	46	>100	100
	3 TCTCGAGCGTTCTC (SEQ ID NO: 72)	16	40	28	41	>100	100
25	4 TCGAGCGTTCTC (SEQ ID NO: 79)	23	15	13	25	>100	80
	5 CTCGAGCGTTCT (SEQ ID NO: 10)	21	14	16	21	95	92
30	6 TCGAGCGTTCT (SEQ ID NO: 4)	10	4	15	35	45	78
	7 TCGAGCGTTC (SEQ ID NO: 116)	6	5	17	40	35	82

8	TCGAGCGTT	1	7	5	7	32	25
	(SEQ ID NO: 117)						
9	TCGAGCGT	1	5	1	13	25	12
	(SEQ ID NO: 91)						
5 10	TCGAGCG	1	1	1	5	9	4
	(SEQ ID NO: 115)						

## C: CpG motifs located at the 5' end of the ODN are most stimulatory

10	1	TCGAGCGTTCTC	12	40	52	59	80	>100
		(SEQ ID NO: 79)						
	2	TCGAGGCTTCTC	6	12	51	61	>100	>100
		(SEQ ID NO: 75)						
	3	TGCTTCGAGCTC	4	3	12	16	20	60
		(SEQ ID NO: 105)						
15	4	GCGAGGCTTCTC	5	12	18	14	>100	>100
		(SEQ ID NO: 127)						
	5	TGCAGCGAGCTC	5	2	4	4	1	16
		(SEQ ID NO: 85)						
	6	CGAGCGTTCTC	<1	<1	1	1	<1	2
20		(SEQ ID NO: 48)						

## D. Optimization of the 5' CpG flanking region

	1	TCGATGCTTCTC	5	12	60	67	100	9
		(SEQ ID NO: 95)						
25	2	TCGAGGCTTCTC	6	12	51	61	160	5
		(SEQ ID NO: 75)						
	3	ACGAGGCTTCTC	3	3	18	23	110	11
		(SEQ ID NO: 34)						
	4	GCGAGGCTTCTC	3	1	18	14	72	6
		(SEQ ID NO: 127)						
30	5	CCGAGGCTTCTC	4	1	15	25	25	7
		(SEQ ID NO: 82)						
	6	TGCTTCGAGCTC	3	1	12	16	60	40
		(SEQ ID NO: 105)						
35	7	TGCAGCGAGCTC	2	1	4	4	16	8
		(SEQ ID NO: 82)						

## E. Optimization of the 3' CpG flanking region

5	1	TCGTTTGTCTC (SEQ ID NO: 93)	8	8	28	31	>100	>100
	2	TCGTATGTACTC (SEQ ID NO: 33)	8	8	26	32	2	33
	3	TCGGATGAGCTC (SEQ ID NO: 29)	6	8	9	20	28	41
	4	TCGAATGCTCTC (SEQ ID NO: 28)	3	5	14	22	6	14
	5	TTGTTCTGTTCTC (SEQ ID NO: 27)	3	4	15	14	19	26
10	6	TTGTTCTGTACTC (SEQ ID NO: 25)	2	4	14	13	15	72
	7	TTGTTCTGAGCTC (SEQ ID NO: 24)	2	4	6	4	6	16
	8	TTGTTCTGAACTC (SEQ ID NO: 24)	2	2	11	5	1	1

Over 200 novel ODN were synthesized, and their ability to activate PBMC from multiple donors examined. PBMC were stimulated for 72 hours in the presence of ODN (1  $\mu$ M added at time 0). Cytokine and antibody secretion in the supernatants were assessed by ELISA, while proliferation was determined by [ $H$ ]<sup>3</sup> uptake. Examples of general findings are presented in this Table. Results are expressed as fold increase over unstimulated cells.

As with D ODN, eliminating the CpG dinucleotide from a K type ODN significantly reduced immune activation (Table 3A, line 3 versus line 4; p ,0.02). Incorporating multiple CpGs in a single ODN increased immune stimulation (Table 3A, lines 1–3). To determine the minimum length of a stimulatory K ODN, nucleotides were sequentially deleted from each end. ODN at least 12 bases long consistently induced strong immune cell activation, whereas shorter ODN were relatively less active (Table IB, lines 1–5 vs lines 6–10). CpG motifs at the 5' end were the most stimulatory (Table 3C, line 2 vs line 3 and line 4 vs line 5), although at least one base upstream of the CpG was required (Table 3C, line 1 vs line 6). Indeed, a thymidine in the immediate 5' position (Table 3D, lines 1 and 2 vs lines 3–5 and line 6 vs



line 7) and a 3' TpT or a TpA (Table 3E, lines 1 and 2 vs lines 3 and 4 and lines 5 and 6 vs line 7) yielded the most active K ODN. Modifications >2 bp from the CpG dinucleotide had relatively less effect on ODN activity.

5

### Example 5

#### Cellular targets of K and D ODN

The phenotype of the cells stimulated to produce cytokine was determined by combined cell surface and intracytoplasmic staining. As seen in Table 4, D ODN selectively stimulated CD3-CD16+CD56+CD14-cells to produce IFN- $\gamma$ , consistent with the direct activation of NK cells.

10

**Table 4**  
**Phenotype of PBMC stimulated by CpG ODN to secrete cytokines**

#### 15 A. Phenotype of PBMC activated by "D" ODN to produce IFN $\gamma$

Cell surface marker	% positive cells
CD16	91
CD56	99
CD3	5
20 CD14	<1

#### B. Phenotype of PBMC activated by "K" ODN to produce IL-6

Cell surface marker	% positive cells
CD83	93
25 CD14	70
CD11c	69
CD19	13
CD16	<1

30 Freshly isolated PBMC were stimulated with K" (8hr) or D (24hr) ODN. Cells were fixed, permeabilized and stained with PE- anti-IL-6 or anti-IFN $\gamma$ . Results are representative of 4-10 experiments.

Without being bound by theory, the effect appears to be direct because  
35 D ODN do not induce a significant increase in IL-12 secretion. Moreover, studies using neutralizing anti-IL-12, which reduce the production of IFN-g by

PBMCs stimulated with PHA (44% p , 0.05) or with bacillus Calmette-Guerin (77%; p , 0.05), did not decrease the IFN- $\gamma$  production induced by CpG-ODN.

By comparison, K ODN stimulated CD14+, CD11c+, and CD83+ cells to produce IL-6, indicating that they were of monocyte/dendritic cell lineage (Table 4B). K ODN also stimulated a fraction of CD19+ B cells to release IL-6.

To confirm these findings, human NK, T, and B cell lines were tested for their responsiveness to K and D ODN. The NK-92 cell line responded exclusively to D ODN by secreting IFN- $\gamma$ , whereas the human RPMI 8226 B cell line was stimulated by K ODN to release IL-6. Non-CpG ODN did not stimulate either cell line.

Thus, as disclosed herein, there are two structurally distinct types of CpG ODN that stimulate different cellular elements of the immune system to mount divergent responses. K type ODN induce monocytes/dendritic cells to produce IL-6 and B cells to proliferate and secrete IgM, whereas D type ODN support NK production of IFN- $\gamma$  (see Table 1). These CpG ODN can be used as vaccine adjuvants, anti-allergens, and immunoprotective agents, as disclosed herein.

The CpG motifs at the center of K and D ODN differ. The optimal K motif contains a thymidine immediately 5' of the CpG, and a TpT or TpA 3' of the CpG. By comparison, optimally active D type ODN contain Pu-Py dimers on each side of the CpG. D ODN also are longer. In one embodiment, the CpG flanking regions are self-complementary. Two dimensional computer modeling further suggested that this self-complementary sequence facilitates the formation of a hairpin loop that exposes the CpG at the apex. Without being bound by theory, this stem-loop structure contributes to the recognition of D ODN because IFN- $\gamma$  production declines when the length or binding strength of the palindrome is reduced (Fig. 2E). Without being bound by theory, the inclusion of poly G at the 3' end of the ODN likely further confers a structural benefit. Alternatively, the poly Gs may improve the efficiency of cellular uptake.

As demonstrated herein, K and D ODNs activate distinct cell types. K ODN activate monocytes and B cells to secrete IL-6, whereas D type ODN stimulate NK cells to secrete IFN- $\gamma$  (Table 1). It is believed that this difference is not due to differential uptake of the ODNs, as monocytes and NK cells take up both D and K ODNs. Without being bound by theory, K and D type ODNs activate their target cells directly, as 1) CpG ODN stimulate cloned cell lines to secrete cytokines; 2) cytokine mRNA appears within minutes of ODN stimulation; and 3) ELISPOT studies show that the CpG ODN induced rapid increase (two to five-fold) in IL-6 and IFN- $\gamma$  secreting PBMC (5 and 18 hour after stimulation, respectively). Moreover, flow cytometric analysis of cells stimulated to secrete IFN- $\gamma$  by CpG ODN were CD3- even after 72 h of stimulation, indicating that the increased IFN- $\gamma$  in supernatants is not due to the secondary activation of T cells.

## Example 6

### Further evidence of Differing Functions of D and K ODNs

#### *Material and Methods*

Oligonucleotides and antibodies: ODN were synthesized at the CBER core facility. Sequences of the CpG ODN used in this study are: 5'-TCGAGCGTTCTC-3' (K23, SEQ ID NO: 79) and 5'-GGtgcatcgatgcaggggGG-3' (D35, SEQ ID NO: 1). The control for K ODN was: 5'-TCAAGTGTCTC-3' (SEQ ID NO: 140) and for D ODN was: 5'-GgtgcatctatgcaggggGG-3' (SEQ ID NO: 141). In this example, bases shown in capital letters are phosphorothioate while those in lower case are phosphodiester. CpG dinucleotides are underlined. All FITC, PE and cychrome labeled Mabs were purchased from Pharmingen (San Jose, CA). All ODNs used in this study contained <0.1 U/mg of endotoxin.

Cell cultures: PBMC from normal donors (provided by the NIH Department of Transfusion Medicine) were isolated by Ficoll-Hypaque density gradient centrifugation (Verthelyi, D, et al., *J. Immunol.* 166:2372-2377, 2000).

Countercurrent centrifugal elutriation was used to isolate monocytes that were >95% pure.  $0.5 - 4 \times 10^6$  cells/ml were cultured in RPMI 1640 containing 5 % FCS, 50 U/ml penicillin, 50 µg/ml streptomycin, 0.3 mg/mL L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 10 mM HEPES and  $10^{-5}$  M 2-mercaptoethanol. Cells were stimulated with ODN for 8 - 72 h with 1 - 3 µM ODN, depending upon the assay.

Analysis of cell proliferation: PBMC were cultured in complete medium plus 3 µM ODN for 72 h. To study B cell proliferation, cells were loaded with 10 nM of CFSE (Molecular Probes Inc., Eugene, OR) as described before (24). Proliferation of CD11c<sup>+</sup> monocytes was monitored by adding 10 µM of BrdU (Pharmingen, San Jose, CA) for the last 18 h of culture. Staining for BrdU was performed as recommended by the manufacturers.

ELISA assays: 96-well microtiter plates (Millipore, Bedford, MA) were coated with anti-cytokine or anti-IgM Ab and blocked with PBS-5% BSA (8). The plates were incubated for 2 h with culture supernatants from PBMC ( $5 \times 10^5$ /ml) that had been stimulated for 8 - 24 h with ODN as described above. IL-6, IFN $\gamma$  and IgM were detected colorimetrically using biotin-labeled antibodies followed by phosphatase-conjugated avidin and a phosphatase specific colorimetric substrate (Verthelyi, D, et al., *J. Immunol.* 166:2372-2377, 2000). The detection limit of the assays was: 6 pg/ml for IFN $\gamma$ , 20 pg/ml for IL-6, and 10 ng/ml for IgM. All assays were performed in triplicate.

Staining for cell surface markers and Intracellular cytokine: Cultured cells were washed in cold PBS, fixed, and stained with fluorescent labeled anti-CD69 (24 h), anti-CD25 (72 h), anti-CD83 (72 h) or anti-CD86 (72 h). To detect intracytoplasmic cytokine, cells incubated with ODN for 8 h were washed, fixed, permeabilized (as per manufacturer's instructions, Caltag, CA) and stained with 4 µg/ml PE-conjugated anti-IL-6 or 2 µg/ml PE-conjugated anti-IFN $\gamma$  (Pharmingen, San Jose, CA) plus various FITC and Cy-Chrome labeled surface markers for 30' at RT. Samples were washed and analyzed

(20,000-40,000 events) on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) after gating on live cells with proper electronic compensation. The data were analyzed using CELLQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA).

5

Analysis of cell-surface binding and internalization of ODN: PBMC ( $4 \times 10^6$ /ml) were incubated with biotinylated ODN (1-3  $\mu$ M) for 10' at 4°C (binding experiments) or 37°C for 1 h (uptake experiments). To detect internalized ODN, surface bound ODN was blocked with 100  $\mu$ g/ml of "cold" streptavidin. After washing, these cells were permeabilized, fixed, and stained with PE-conjugated streptavidin (1  $\mu$ g/ml) plus FITC or Cy-Chrome conjugated cell surface markers.

10

Confocal Microscopy Elutriated monocytes ( $4 \times 10^6$ /ml) were incubated with Cy-3 or FITC labeled K and/or D ODN at 37°C for 1 h. The cells were washed, and mounted using the Prolong antifade kit (Molecular Probes, Oregon, USA). Subcellular localization of Cy3 and FITC labeled ODN was determined by confocal microscopy under 1000 x magnification (LSM5 PASCAL, Carl Zeiss In., Thornwood, NY).

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Binding and internalization of CpG ODN.

The ability of human PBMC to bind and internalize CpG ODN was examined using fluorescent-labeled K23 and D35 ODN. Both types of ODN bound rapidly to the surface of virtually all human monocytes at 4°C. A significant fraction of B lymphocytes (20 - 45%) and NK cells (~10-20%) also bound these ODN. Simultaneous staining with K and D ODN showed that the same cells were binding both types of ODN. In contrast, interaction with T cells barely exceeded background levels.

25

To monitor internalization, PBMC were incubated with biotin-labeled ODN for 60' at 37° C. Surface-bound ODN was blocked with excess strepavidin, and internalized ODN detected by staining fixed, permeabilized cells with FITC-avidin. The fraction of monocytes, B lymphocytes and NK

30

cells that internalized K23 and D35 ODN was similar to the fraction of each cell type that bound these ODN. Similar results were obtained using other D and K ODN. No internalization was observed when cells were incubated with ODN for 10' at 4°C, suggesting that ODN uptake involves metabolic activity.

5           The ratio of membrane bound : internalized ODN was compared. Based on differences in mean fluorescence intensity, it was calculated that target cells internalized approximately half of the ODN that had bound to their cell surface. For all cell types, the absolute magnitude of D ODN uptake exceeded that of K ODN. For example, the amount of labeled D ODN that  
10 bound to and was taken up by monocytes exceeded that of equimolar K ODN by ~2-fold throughout the functional concentration range of these agents ( $p < 0.001$ ). To achieve equivalent levels of binding and uptake required that "D" ODN be used at a 4-fold lower concentration than K (e.g. 0.75 vs 3.0  $\mu\text{M}$ ).

          The intracellular localization of these two types of ODN was examined  
15 by confocal microscopy of labeled monocytes. K and D ODN largely occupied discrete areas within the same cell, although there was a limited degree of co-localization. D ODN largely occupied punctuated vesicles, whereas K ODN were more diffusely distributed, staining the nucleus as well as cytoplasmic vesicles. This difference in localization was associated with the presence or  
20 absence of a poly G tail, since control (non-CpG) ODN with a poly G tail showed the same distribution pattern as did D ODN. In contrast, the fluorescent dyes used did not influence distribution, since switching dyes had no effect on ODN localization pattern.

## 25   *Differential effect of K versus D ODN on B cell function*

          Whole PBMC were treated with optimal concentrations of K23 and D35 ODN. K ODN rapidly activated CD19<sup>+</sup> B cells, reflected by a significant increase in the expression of the CD69 early activation marker and the CD25 late activation marker ( $p < 0.001$ ). K ODN also triggered a >10-fold increase in  
30 B cell proliferation ( $p < 0.05$ ), a >10-fold increase in IgM production ( $p < .01$ ), and a 5-fold increase in the number of B cells secreting IL-6 ( $p < 0.001$ ). The effect of K23 exceeded that of D35 (and of a control for the K type ODN of the

same structure but lacking the critical CpG motif) by >10-fold in each of these functional assays. D ODN were not entirely inactive, however, since they induced a modest increase in CD25 and CD69 expression by CD19<sup>+</sup> B cells.

5 *Differential effect of K versus D ODN on NK cells*

NK cells were identified by their expression of the CD16 surface marker. D ODN stimulated approximately 25% of these cells to increase expression of CD25 and CD69 ( $p < 0.001$ ). Consistent with previous studies, D ODN also triggered a significant increase in IFN $\gamma$  secretion by NK cells ( $p < 0.05$ ). By comparison, neither K ODN nor a non-CpG control for the D ODN significantly stimulated IFN $\gamma$  production (see also Table 1). K23 did induce a modest increase in the number of NK cells expressing CD25 and CD69 ( $p < 0.05$ ). None of these ODN induced NK cells to proliferate.

15 *Differential effect of K versus D ODN on monocytes*

K and D ODN had disparate effects on purified monocytes. K23 stimulated CD14<sup>+</sup> monocytes to proliferate ( $p < 0.05$ ) and secrete IL-6 ( $p < 0.001$ ), while D35 had no effect in these assays (Table 5). Instead, D (but not K) ODN stimulated monocytes to mature into CD83<sup>+</sup>/CD86<sup>+</sup> dendritic cells (DC) ( $p < 0.001$ , Table 5). The divergent effects of K versus D ODN on monocytes persisted throughout the physiologic concentration range of both types of ODN, and was observed using a variety of D and K ODN, indicating that these differences were not due to variation in ODN binding or uptake. Although both types of ODN increased CD69 and CD25 expression, D ODN up-regulated these activation markers in monocytes significantly more effectively ( $p < 0.001$ , Table 5).

**Table 5**  
**Effects of D ODN**

		% of Cells Expressing/Producing				
ODN type	CD25	CD 69	IL-6	L-6 (ng/ml)	Proliferation (SI)	DC maturation
K23	32.8 $\pm$ 1.1	39.7 $\pm$ 5.3	24.1 $\pm$ 2.4	2.0 $\pm$ 0.3	7.0 $\pm$ 1.4	3.5 $\pm$ 0.3
D35	48.6 $\pm$ 7.5	82.1 $\pm$ 2.4	4.6 $\pm$ 0.3	0.8 $\pm$ 0.1	1.0 $\pm$ 0.5	23.7 $\pm$ 2.0
K23 + D35	44.7 $\pm$ 13.9	53.8 $\pm$ 12.5	ND	2.2 $\pm$ 0.1	2.8 $\pm$ 1.7	5.2 $\pm$ 0.8
"K" control	21.8 $\pm$ 2.2	30.2 $\pm$ 2.5	5.1 $\pm$ 0.6	<.03	2.1 $\pm$ 1.6	1.8 $\pm$ 0.1

PBMC or elutriated monocytes were stimulated with 3  $\mu$ M of ODN for 8 - 72 h. The percent of CD14<sup>+</sup> monocytes induced to secrete IL-6 was determined by intracytoplasmic staining. Due to the down-regulation of CD14, CD11c was used to monitor the expression of CD25 and CD69 by stimulated cells. The percent of CD83<sup>+</sup>/CD86<sup>+</sup> dendritic cells in culture was determined after 72 h. IL-6 levels in culture supernatants were determined by ELISA, while proliferation was evaluated by BrDU incorporation. Results represent the mean  $\pm$  SD of 5 independent experiments.

#### *Competition between K and D ODN at the single cell level*

The above findings suggested that monocytes responded differently to stimulation by K versus D ODN. There are two possible explanations for this observation: either i) these two types of ODN were triggering the same cells to mount distinct types of immune response or ii) K and D ODN were acting on different subpopulations of monocytes. The latter explanation seemed unlikely, given that confocal microscopy showed that the same cells were binding and internalizing both types of ODN.

To clarify this situation, monocytes were treated simultaneously with D35 plus K23. At optimally stimulatory concentrations, these ODN did not cross-compete for uptake or binding. Yet when their function was analyzed, co-administration of K ODN reduced the ability of D ODN to trigger monocyte differentiation by 70% ( $p < 0.001$ ). The inhibitory effect of K ODN on the activity of D ODN was sequence specific and concentration dependent, since control non-CpG ODN did not significantly interfere with the activity of D ODN. Conversely, D ODN significantly reduced the ability of K ODN to induce monocytes to proliferate ( $p < 0.05$ ). As above, the inhibitory effect of D on the activity of K ODN was sequence specific and concentration dependent.

A very different pattern emerged when B and NK cells were studied. In these cells, the co-administration of D with K ODN was not inhibitory. Rather, the ability of K ODN to stimulate B cells to proliferate and secrete IL-6 and



IgM was unaffected by the presence of D ODN, and the ability of D ODN to stimulate NK cells to secrete IFN $\gamma$  was not reduced by inclusion of K ODN.

### Example 7

#### 5 D ODN enhance tumor lysis.

Reports of tumor regression following systemic bacterial infection stimulated research into immune adjuvants as a strategy for tumor treatment. Immunostimulatory oligodeoxynucleotides (ODNs) containing the CpG motif have been demonstrated to induce the production of various cytokines, natural killer cells, monocytes, lymphocytes and dendritic cells (see above). Thus, short-term bladder transitional cell carcinoma (TCC) cultures were treated with CpG ODN immunostimulated PBMC and the cultures were analyzed for evidence of tumor lysis.

#### 15 *Materials and Methods:*

Papillary TCCs from 4 patients were cultured. Peripheral blood mononuclear cells (PBMCs) were obtained from each patient. In matched cultures, the tumor cells were treated with PBMCs stimulated *in-vitro* with :1) CpG ODN in the presence or and absence of tumor cells, 2) bacillus Calmette-Guerin (BCG) stimulated PBMCs (positive control and standard of care), 3) unstimulated PBMCs, and/or 4) BCG or CpG ODN in the absence of the patient's PBMCs (negative controls).

The morphologic and lytic characteristics of these cultures were compared with those of untreated matched tumor cell cultures. A chromium based CTL assay method was used for quantitative comparison of tumor lysis.

#### *Results*

PBMCs that were stimulated *in vitro* with CpG ODN type D in the presence of tumor cells lysed 30-70% of the tumor cells from 3 of 4 patients.

**Table 6**

	Percent lysis
PBMC+TCC	4+5
PBMC+TCC+D ODN	41+15
PBMC+TCC+K ODN	3+3
PBMC+TCC+ CONTROL ODN	3+2
PBMC+TCC+BCG	71+24

Thus, CpG ODN immunostimulation was associated with enhancement of tumor lysis comparable to that of BCG. K ODN did not enhance tumor lysis. Similarly, tumor lysis was not enhanced in the absence of CpG ODN (the negative control).

**Example 8****15      Primates Provide a Model System for the Study of CpG ODN**

Primate PBMCs respond in a similar manner as human PBMCs to CpG ODN. To demonstrate the similarities, PBMC from normal human donors and Rhesus macaques were stimulated with a panel of K, D or control ODNs, and the IL-6 and IFN- $\alpha$  levels produced were monitored (Figs. 4 and 5). In both human and monkey PBMC cultures, D ODNs induced the secretion of IFN- $\alpha$ , while K ODNs induced cells proliferation and IL-6 production. Thus, rhesus macaques provide a accurate model system for studying the effects of ODNs *in vitro*.

In order to establish that rhesus macaques were a model system for *in vivo* experiments, macaques were treated an antigen (OVA) and with either D type (D19 and D29) or K type (K3 and K23) ODNs, or with a control ODN (A23). The anti-OVA IgG titers in the group that received D ODN were significantly higher (Fig. 6), thus demonstrating that D ODN can increase the immune response to a protein antigen *in vivo*. Thus, rhesus macaque monkeys provide an *in vivo* model system to study the effects of ODNs.

### Example 9

#### Effect of D ODN on PBMC from Primates

As disclosed in this example, rhesus macaques provide a useful model for assessing the activity of CpG ODN *in vivo*. *In vitro* studies established that PBMC from rhesus macaques responded to the same panel of K and D ODN that were highly active on human PBMC. CpG ODN were co-administered with a mixture of ovalbumin plus alum. The ODN significantly boosted the antigen-specific IgG response of macaques, with D being superior to K ODN. A cutaneous leishmania infection model was then used to examine whether CpG ODN could boost protective immunity in primates. The nature, severity, duration and histopathology of the cutaneous disease caused by *L. major* in macaques is quite similar to that in humans (for example see. Amaral et al., *Exp Parasitol* 82:34, 1996). Results indicate that D ODN significantly improve the protection conferred by co-administered heat-killed leishmania vaccine (HKLV).

#### Materials and Methods Utilized

Rhesus monkeys: Healthy 3 year old female rhesus macaques (*M. mulata*) were obtained from the FDA colony in South Carolina. All studies were ACUC approved and were conducted in an AAALAC accredited facility. Animals were monitored daily by veterinarians. No systemic or local adverse reactions to CpG ODN, OVA or HKLV immunizations were observed. Treatments were administered and peripheral blood samples obtained from ketamine anesthetized animals (10 mg/kg, Ketaject, Phoenix Pharmaceuticals, St Joseph, MD).

Vaccination groups and protocol: Two *in vivo* studies were conducted. 1) 3 monkeys/group were immunized subcutaneously (s.c.) and boosted 12 weeks (wk) later with a mixture of 4 µg of ovalbumin, 250 µg ODN and 125 µg of alum (Rehydragel HPA, Reheis, Berkeley Heights, NJ). 2) 5-6 monkeys/group were immunized s.c. and boosted 4 weeks later with 250 µg of GMP grade HKLV (Biobras, Montes Claros, Brazil) plus 125 µg of alum, as

previously described (27). The HKLV was administered alone, or combined with 500 µg of ODN. Preliminary studies established that this dose of ODN was active *in vivo* and well-tolerated. Animals were exposed to non-viable *L. amazonensis* metacycle promastigotes on week 8, a treatment that induced no disease and no change in antibody titer or proliferative response to *Leishmania* antigens when compared to control animals. Animals were challenged on the forehead on week 14 with 10<sup>7</sup> viable *L. major* (WHOM/IR-/173) metacyclic promastigotes i.d. The monkeys developed a typical self-limited *in situ* lesion characterized by erythema, induration, and ulceration. Lesion size, which reflects the severity of infection, was measured weekly.

Oligodeoxynucleotides: ODN were synthesized by the CBER Core Facility. All ODN had less than <0.1 EU of endotoxin per mg of ODN as assessed by a Limulus amoebocyte lysate assay (QCL-1000, BioWhittaker). The following ODN were used in this work:

D19: GGTGCATCGATGCAGGGGGG (SEQ ID NO: 1)  
D29: GGTGCACCGGTGCAGGGGGG (SEQ ID NO:2)  
D35: GGTGCATCGATGCAGGGGGG (SEQ ID NO: 1)  
D122: GGTGCATTGATGCAGGGGGG (SEQ ID NO: 53)  
K3: ATCGACTCTCGAGCGTTCTC (SEQ ID NO:3)  
K123: TCGTTCGTTCTC (SEQ ID NO:92)  
K23: TCGAGCGTTCTC (SEQ ID NO: 79)  
K163: TTGAGTGTTCTC (SEQ ID NO:109)  
AA3M: GGGCATGCATGGGGGG (SEQ ID: 142)

Mononuclear cell preparation: Human and monkey mononuclear cells were isolated by density gradient centrifugation of PBMC over Ficoll-Hypaque as described (see above). Cells were washed 3 times and cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS (FCS), 1.5 mM L-glutamine and 100 U/ml of penicillin/streptomycin at 5 x 10<sup>5</sup> cells/well in the

presence of 3  $\mu$ M ODN. Supernatants were collected after 72 hours and tested by ELISA for cytokine and antibody levels.

ELISA: 96-well microtiter plates (Millipore Corp., Bedford, MA) were coated with Abs that cross-reactively recognized human and macaque IL-6 (R&D, Minneapolis, MN), IFN- $\alpha$  (PBL Biomedical Laboratories, New Brunswick, NJ), and IgG (Boehringer-Mannheim Biochemicals, Germany). The plates were blocked with PBS-5% BSA. Culture supernatants from PBMC cultures were added, and their cytokine content quantitated by the addition of biotin-labeled anti-cytokine Ab followed by phosphatase-conjugated avidin and phosphatase-specific colorimetric substrate. Standard curves were generated using known amounts of recombinant human cytokine or purified Ig. All assays were performed in triplicate. Titers of antibodies to ovalbumin in sera were assayed on OVA-coated plates.

ELIspot: The number of PBMC secreting IFN- $\gamma$  in response to soluble leishmania antigen (SLA) were determined by ELIspot as described (Hagiwara *Cytokine* 7:815, 1995). Briefly, Millipore 96-well filtration plates (Millipore Corp., Bedford, MA) were coated overnight at 4° C with 1  $\mu$ g/ml of anti-human IFN- $\gamma$  antibodies (Clone GZ4, Alexis, San Diego, CA) in PBS and then blocked with PBS-5%BSA for 2 hr. The plates were overlaid with  $5 \times 10^5$  cells/well (1-2 series/ monkey) and incubated at 37° C in a humidified 5% CO<sub>2</sub> in air incubator for 18 hr in the presence of 25  $\mu$ g soluble leishmania antigen (SLA). The plates were then washed with water-0.025% Tween and overlaid with biotin conjugated anti-human IFN- $\gamma$  (clone 76-B-1, Mabtech, Sweden). After 2 hr the plates were washed again and then overlaid with alkaline phosphatase-conjugated streptavidin. Spots were visualized by the addition of 5-bromo-4-chloro-3-indolyl phosphate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) and counted using the KS ELIspot Imagine System (Carl Zeiss, Inc., Thornwood, NY).

Cell proliferation assay:  $10^5$  PBMC/well were incubated with 3  $\mu$ M of ODN for 68 h, pulsed with 1  $\mu$ Ci of [ $^3$ H] thymidine and harvested 4 h later. All assays were performed in triplicate.

- 5        Statistical Analysis: Statistically significant differences were determined using a 2-tailed non-parametric ANOVA with Dunnett's post test analysis. Differences in lesion sizes were tested by repeated-measures ANOVA using the Proc Mixed procedure from the Statistical Analysis System (SAS).

RESULTS:

10        ***Response of PBMC from human and non-human primates to K and D ODN***

- As disclosed herein, human PBMC respond to two structurally distinct classes of CpG ODN. D type ODN triggered the secretion of IFN- $\gamma$  and IFN- $\alpha$  whereas K ODN induced human PBMC to proliferate and secrete IL-6 and IgM (Table 1). D and K ODN that strongly activated human cells were tested for their ability to stimulate PBMC from rhesus macaques.

- The response of rhesus PBMC to "D" ODN was evaluated on the basis of IFN- $\gamma$  production. Results show that macaque PBMC are activated by the same D ODN that stimulate human PBMC ( $p < 0.002$ , Fig. 4). In contrast, neither K ODN, nor control ODN that are structurally similar to D ODN but lack the critical CpG dinucleotide, had this effect.

- Proliferation and IL-6 secretion were used to compare the response of macaque and human PBMC to K ODN. PBMC from both species were stimulated by K ODN to proliferate ( $p < 0.002$ ) and secrete IL-6 ( $p < 0.01$ ), whereas controls of the same structure as K ODN but lacking the critical CpG motif failed to trigger immune stimulation. The results demonstrated that the pattern of reactivity of PBMC from rhesus macaques ( $N = 20$ ) and humans ( $N = 8-20$ ) to K and D ODN is similar.

- Mixtures of ODN were identified that strongly stimulated PBMC from all human donors were utilized in further experiments. These mixtures were tested on PBMC from macaques and found to be uniformly active (Fig. 5). Subsequent *in vivo* studies were conducted with these ODN mixtures.

**Adjuvant activity of CpG ODN *in vivo***

Previous studies in mice showed that CpG ODN could boost the immune response to a co-administered protein antigen (such as ovalbumin).

- 5 This effect was amplified by adding alum to the mixture of CpG ODN plus antigen (for example see Klinman, *Vaccine* 17:19, 1999). Building on these results, macaques were immunized and boosted with a mixture of OVA, alum, and ODN (see Fig. 6). Animals immunized with mixtures containing DODN increased their IgG anti-OVA response by 470-fold after primary ( $p < 0.05$ ) and
- 10 by 600-fold after secondary ( $p < 0.01$ ) immunization. By comparison, K ODN boosted the IgG Ab response by 7-fold after primary and 35-fold after secondary immunization when compared to pre-treatment values ( $p < 0.05$ ). Macaques immunized with OVA plus control ODN generated only a 4-fold increase in anti-OVA titer. These findings indicate that D ODN are particularly
- 15 effective at boosting the antigen-specific humoral response to a co-administered antigen.

- CpG ODN that activate human immune cells *in vitro* are only weakly immunostimulatory in mice. The results disclosed herein document that rhesus macaques are a relevant model for examining the *in vivo* activity of
- 20 CpG ODN. PBMC from macaques mirrored the response of human PBMC in their response to both "K" and "D" ODN. At the immunostimulatory doses used in this study, neither type of ODN triggered any adverse events. *In vivo*, broadly immunostimulatory mixtures of K and D ODN boosted antigen-specific IgG responses in macaques immunized with OVA and increased IFN- $\gamma$
- 25 production in animals vaccinated with HKLV. In addition, as described below, D ODN significantly increased the protective response elicited by a co-administered vaccine.

### Example 10

#### Effect of CpG ODN on the Immunogenicity and Protective Efficacy of Heat-Killed Leishmania Vaccine (HKLV)

Previous human clinical trials showed that HKLV was safe, but poorly immunogenic (Handman et al., *Clin Microbiol. Rev.* 14:229, 2001). In addition, HKLV combined with alum and IL-12 induces short-term protective immunity in rhesus macaques (Kenney et al., *J Immunol* 163:4481, 1999), and CpG ODN plus alum increased the immune response to the hepatitis B vaccine in cynomolgus monkeys (Hartmen et al., *J Immunol* 164:1617, 2000).

Macaques were immunized and boosted with a mixture of HKLV, alum and CpG ODN. PBMC from these animals were isolated 10 days post boost and re-stimulated *in vitro* with leishmania antigen for 72 hr. As seen in Fig 7, both K and D ODN significantly increased the number of PBMC triggered to secrete IFN- $\gamma$  ( $p < 0.05$ ). In contrast, animals immunized with alum-adsorbed HKLV alone showed no increased IFN- $\gamma$  production when compared to unimmunized controls.

The critical measure of an antigen/adjuvant combination is its ability to induce protective immunity. Vaccinated animals were therefore challenged with  $10^7$  *L. major* metacyclic promastigotes. Animals vaccinated with HKLV-alum alone developed typical cutaneous lesions with a peak surface area of  $300 \pm 60$  mm<sup>2</sup> 26 days after challenge (Fig. 7). Monkeys vaccinated with HKLV-alum plus K ODN developed lesions of similar size, although the peak lesion formation was slightly delayed. Animals immunized with HKLV-alum plus "D" ODN had significantly smaller lesions (maximal size  $80 \pm 13$  mm<sup>2</sup>,  $p < 0.05$ ), consistent with a reduced parasite burden.

All animals treated with CpG ODN, either alone or with antigen, remained healthy and active throughout the study. No hematologic or serologic abnormalities were observed 3 days or 9 months after injection and no weight loss or change in behavior were detected following administration of CpG ODN at therapeutic doses.

As disclosed herein, cutaneous infection of macaques with *L. major*



provides a means for testing the protective efficacy of CpG ODN - vaccine combinations. The nature, severity and duration of the cutaneous disease caused by *L. major* in macaques is quite similar to that in humans. The leading leishmania vaccine candidate (HKLv) has proven safe but poorly immunogenic in clinical trials (Handman, *Clin Microbiol. Rev.* 14:229, 2001). Co-administration of both D and K ODN with this alum-adjuvanted HKLV vaccine significantly increased the number of PBMC triggered to secrete IFN- $\gamma$  when stimulated with leishmania antigen *in vitro*. However, the critical test of any vaccine/adjuvant combination is its ability to induce protective immunity. Results show that the cutaneous lesions of macaques vaccinated with HKLV plus "D" ODN were significantly reduced when compared to HKLV-alum alone. A reduction in lesion size correlates with a reduced parasite load. Thus, without being bound by theory, the findings suggest that the ability of D ODN to stimulate IFN- $\gamma$  and IFN  $\alpha$  production while promoting the maturation of antigen presenting cells can be useful for the induction of a protective response against leishmania. In addition, without being bound by theory, the findings suggest that the ability of D ODN to stimulate IFN- $\gamma$  and IFN  $\alpha$  production while promoting the maturation of antigen presenting cells can be useful as a vaccine adjuvant.

All of the references cited herein, including patents, patent applications, and publications, are hereby incorporated in their entireties by reference.

While this invention has been described with an emphasis upon preferred embodiments, it will be obvious to those of ordinary skill in the art that variations of the preferred embodiments may be used and it is intended that the invention may be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications encompassed within the spirit and scope of the invention as defined by the following claims.